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Ecological interactions of rhizobia and their effects on the nitrogen nutrition of field pea

by

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Declaration of authenticity

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

07-January-2020 

DATE **SIGNATURE**

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Abbreviations

ANOVA	Analysis of Variance
BLAST	Basic local alignment search tool
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DF	Degrees of freedom
DNA	Deoxyribonucleic acid
DPI	Department of Primary Industries
ERIC	Enterobacterial repetitive intergenic consensus
ITS	Internal transcribed spacer
IV	Indicator value
NSW	New South Wales
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction
PCoA	Principal coordinate analysis
PerMANOVA	Permutational multivariate analysis of variance
ppm	Parts per million
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid

Abstract

Rhizobial nitrogen (N) fixation is an important source of nitrogen for most legume crops. The amounts of nitrogen added to soils around the world via rhizobial fixation exceeds approximately 20 million tonnes annually. Moreover, rhizobial N fixation has been estimated to replace millions of tonnes of inorganic fertilizer and stimulate other microbial processes like decomposition by adding organic nitrogen into soils. However, rhizobial N fixation is limited in many systems due to several abiotic and biotic factors. For example, having ineffective resident rhizobial populations in soils is a major biotic factor that limits N fixation. Development of effective N fixing rhizobial strains as commercial inoculants is a well-practiced technique in legume industry to enhance plant N nutrition. The success of these commercial inocula under field conditions is still challenging due to the unfavourable competition for nodulation imposed by the ineffective resident populations in field soils. There is a gap between the accurate knowledge on overcoming this inter-strain rhizobial competition and exploiting maximum benefits from rhizobial N fixation. One of the major objective for this study was to examine the extent of the symbiotic interactions of multiple *Rhizobium leguminosarum* strains in a single field pea root system. Then to explore the interactions between competitor rhizobial strains and a commercial inoculant and also to examine whether these symbiotic interactions could significantly affect the overall N nutrition of field pea.I also considered these rhizobial interactions in the context of abiotic stress conditions which could constrain symbiotic N fixation. Among the major abiotic environmental stresses, drought is one of the key abiotic constraints in Australian agricultural systems due to lack of rainfall for prolonged periods of time. Water stress disrupts plant growth and function and reduces populations of effective N fixing rhizobia in soils. Therefore, for this thesis, I investigated whether drought could alter the interactions between commercial (WSM1455) and competitor strains of *Rhizobium leguminosarum* and impact overall N nutrition of field pea hosts (*Pisum sativum* L).

In order to gain more knowledge on how the strength of interactions among strains of rhizobia can affect field pea N nutrition, my work evaluated the efficiency of multi-strain *R. leguminosarum* combinations compared to the efficiency of individual rhizobial strains (chapter 2). I developed inoculant combinations using pairs of rhizobial strains and they differed in their degree of genetic similarity. I predicted the rhizobial genetic similarity as a proxy for ecological similarity and strength of competition. I have observed that nodule number of pea plants was greater for distantly related pairs of rhizobial strains. Strain combinations had ~20% more N fixed in pea plants compared to single strain inoculations. This pattern was consistent across

distantly and closely related pairs in chapter 3 wherethe fixed N amounts of strain combinations were ~20%- 50% higher compared to the amounts fixed by the individual strains on their own.The findings of this work provided evidence for synergistic interactions of rhizobial combinations over single strain inoculations to improve host N fixation. These outcomes showed the importance of exploring the nature of interactions of rhizobial strains when they were developed into combinations.

Chapter 2 focused on interactions among strains inoculated to whole root systems but was not able to differentiate whether these effects were due to strains interacting within individual nodules associated with those root systems. To address this, I examined whether field pea plants could contain mixed infected nodules with two rhizobial strains and estimated the extent that interactions affected nodulation and N fixation (chapter 3). I developed a plate-based plant growth experiment and inoculated combinations of *R. leguminosarum* directly onto roots, to ensure strains were located together in space, and included single-strain inoculations as controls. Mixed nodule infection was only infrequently observed in this field pea-*R. leguminosarum* system regardless of rhizobial genetic similarity. As in chapter 2, I observed that distantly related rhizobial strains resulted in significantly higher nodulation and N fixation. These findings emphasized that genetic similarity might be good for initial screening of combinations of strains for further testing, but the characteristics of the individual strains and how they interact with other specific strains is ultimately the most important. Thereby multi-strain inocula production could carefully select the efficient and non-competing rhizobial strains to gain maximum N benefits. I then explored whether these interactions could be altered in stressful environments (here focusing on drought) and among field pea cultivars that differ in drought-tolerance (chapter 4). In this case, I focused in particular on interactions between the commercial inoculant *R. leguminosarum* WSM1455 and competitor strains. A pot experiment was carried out having well-watered and reduced watering treatment. WSM1455 inoculant on its own produced larger nodules and more N fixation. In contrast, WSM1455 was less effective in nodulation, N fixation and enhancing plant biomass when it was inoculated with competitor strains. This effect was generally consistent across the two watering treatments: drought conditions, nodulation, N fixation and plant biomass was reduced regardless of the rhizobial inoculant type. Among two pea cultivars used in the study, cultivar Wharton gained more fixed N than cultivar Twilight regardless of the watering condition.

Extracting and sequencing of bacterial DNA from pea root nodules showed the presence of both rhizobial and non-rhizobial endophyte (NRE) bacteria in earlier chapters, but more work is

needed to determine whether these NREs varied in frequency in relation to the strains of rhizobia present. To determine the compositional shifts of these NRE communities among different rhizobial inoculation treatments and drought, I examined the diversity and community composition of NREs in nodules inoculated with WSM1455 and competitor strains under well-watered and water-stressed conditions (chapter 5). NRE communities were affected by drought, but this effect was dependent on the type of rhizobial inoculation treatment. While my work did not address the functions that these NREs may have played in nodules, more work is needed to identify their possible benefits in plant growth promotion for exploitation in commercial products.

Rhizobial N fixation is an important source of gaining nitrogen in sustainable agriculture. My work demonstrated that the N fixing efficiency of a commercial rhizobial strain could be restricted by intense competition from ineffective resident rhizobial populations and environmental stresses such as drought. Therefore, production of drought-resistant commercial inocula which can also persist in soils to overcome resident rhizobial competition could benefit legumes with increased N benefits. Using multi-strain rhizobial inoculants over single strain inoculants could be promoted for better N fixing outcomes along with additional field-based testing of interactions among strains of rhizobia as well as other bacteria intimately associated with nodules.

Chapter 1: General Introduction

1.1 Importance of the legume-rhizobium symbiosis

Inadequate supply of nitrogen (N) for plants in low N soils can be an important factor limiting plant growth and productivity (Pell et al., 1995). The persistence of N limitation can be caused by inaccessibility of nitrogen in high energy triple bonds between nitrogen molecules in its gaseous form (Galloway and Cowling, 2002) and low inputs of nitrogen by natural abiotic processes like lightning (Bobbink et al., 2010). Also, there can be major anthropogenic activities that lead to loss of nitrogen from soils such as tillage, removal of harvest and leaching (Vitousek et al., 2002). Some plants overcome N limitation via beneficial symbiotic interactions with rhizosphere microbes (Oldroyd et al., 2011). One such interaction is the legume-rhizobium symbiosis, where atmospheric nitrogen (N_2) is converted to ammonia/ammonium (NH_3/NH_4^+) by rhizobial bacteria using the nitrogenase enzyme complex in root nodules (West et al., 2002).

Rhizobial N fixation represents a significant contribution to agricultural productivity (Poole et al., 2018). Economically important leguminous crops are estimated to assimilate 21.45 Tg N yr^{-1} of biologically fixed nitrogen globally (Herridge et al., 2008, Peoples et al., 2009). In Australia, biological nitrogen fixation has been estimated to replace three million tonnes of inorganic nitrogen fertilizer per year. *Pisum sativum* L. (Field pea) is one of the most important legume crops in Australia, with an average annual production of 0.3 million tonnes (Poblaciones and Rengel, 2016). Furthermore, the production of field pea in Australia is increased substantial, with yields of 204,000 tonnes in Victoria and Tasmania and 68,000 tonnes in New South Wales (NSW) (five-year average to 2016/2017) (GRDC, 2017). Moreover, Australian field pea industry is expanding with 55% of field peas produced being exported to Asian countries.

1.2 Why is the rhizobial symbiosis limited in many agricultural soils?

Rhizobial nitrogen fixation is limited in many legume production systems (Thrall et al., 2011). The presence of ineffective resident rhizobial populations is one of the major biotic factors causing poor crop production in many agricultural fields (Giller et al., 1989). Resident rhizobia can be defined as naturalized rhizobia through past inoculations and/or rhizobial populations native to a soil/field (Mothapo et al., 2013). Not all the rhizobia colonizing host plants fix N effectively (Denison et al., 2003) and some rhizobial strains fix little or no nitrogen in root nodules (Kiers et al., 2007, Ratcliff et al., 2008). In most cases, resident rhizobial populations found in field soils vary in effectiveness and and may be dominated by ineffective N fixers

(Peoples et al., 2001). In a previous study, Drew and Ballard (2010) investigated the nodulation and N fixation efficiency of four pure resident rhizobium isolates collected from *Trifolium subterraneum* L. (subterranean clover) grown field soils across Australia. They have observed that some genotypes of subterranean clover had very low number of nodules with less N fixation with resident strains compared to the commercial inoculant *R. leguminosarum* bv. *trifolii* WSM1325.

Inoculation of leguminous crops with effective N fixing rhizobial strains is therefore a widespread industrial practice for maximizing crop yield (Yates et al., 2005). However, in many cases, commercially produced inoculant strains resulted in poor nodulation due to the competitive dominance of resident rhizobial populations under field conditions (Labandera and Vincent, 1975, Jenkins et al., 1954). For example, Denton et al., (2003) observed competitive dominance of two field rhizobial strains (isolated from Roseworthy field site, South Australia) over the commercial inoculant *Rhizobium leguminosarum* bv. *trifolii* in the nodules of *Trifolium alexandrinum* (Egyptian clover). The production of commercial rhizobial inoculants generally focusses on important rhizobial traits such as successful nodulation and effective nitrogen fixation (Rodríguez Blanco et al., 2010, Dwivedi et al., 2015) under field conditions.

The symbiotic interaction between rhizobia and their host plants is tightly co-evolved and specific (Denison and Kiers, 2004a, Masson-Boivin et al., 2009, Simms and Taylor, 2002). For example, Liu et al., (2012) have examined that *Galega officinalis* and *Hedysarum coronarium* only form effective nodules with their respective symbionts, *Rhizobium galegae* and *R. sullae*. Therefore, these legumes are known as highly specific hosts to their rhizobial symbionts (Andrews and Andrews, 2017). Further, Álvarez-Martínez et al., (2009) explored that *Rhizobium leguminosarum* nodulating *Vicia faba* and *Vicia sativa* from different continents (found in European, American and Asian countries) belonged to a clade with minimal divergence from one another due to restrictive plant hosts. This study analysed core and symbiotic genes of rhizobial strains revealing that *Vicia faba* and *Vicia sativa* are restrictive hosts. Both rhizobia and plant have evolved strategies to recognize each other (Dakora, 2003). Plant roots release flavonoids as signals to attract their nitrogen fixing bacteria (Phillips, 1992). As a response, nod factors (lipo-chito-oligosaccharide molecules) are produced by the rhizobia creating their pathway to plant roots (Ramu et al., 2002). The *nod* genes of rhizobia are known as the major determinants of host specificity (Andrews et al., 2018). Rhizobia enter roots via root hairs through a special structure called an ‘infection thread’ and then nodules are formed (Andrews ans Andrews, 2017). The majority of rhizobial cells change their cell morphologies

inside this infection thread. Rhizobia within the nodule can differentiate into a nitrogen-fixing state called bacteroid which resembles a swollen enlarged version of the normal rod shaped rhizobial cell (Oono et al., 2009). Bacteroids are able to express the genes responsible for the nitrogen fixation such as *nif* genes (Checcucci et al., 2016). It is also important to note that rhizobial symbiosis genes (*nodABC* and *nif*) can be differentiated from housekeeping genes and are often found in plasmids or symbiotic islands in rhizobial cells (Andrews et al., 2018). Despite the specificity of rhizobia to legumes, there is variation within species of rhizobia that might be functionally important, both for nodulation and for nitrogen fixation (Carelli et al., 2000). Therefore, several aspects of rhizobial ecology and evolution, particularly with respect to intraspecific variation among rhizobial strains, require further clarification.

Drought, or prolonged periods of low soil water availability is an important factor in limiting the nodulation process in legumes (Boonkerd and Weaver, 1982). Plants grown in water-limited soils have very low rhizobial N fixation (Guilioni et al., 2003). In most of the Australian legume growing regions (southern regions of Australia, Victoria, NSW and Tasmania) annual precipitation patterns are unreliable where they can vary from more than 750 mm to less than 250 mm (Williams et al., 2002). Water stress can cause disruption of both plant and rhizobial processes (Hungria and Vargas, 2000). A significant decline in rhizobial numbers can be observed in many water-limited legume fields in Australia which in turn reduces effective nodulation (Slattery et al., 2001). Further, drought disrupts plant photosynthetic activities (Gil-Quintana et al., 2013) and negatively affects plant carbon metabolism, protein synthesis, amino acid metabolism, and cell growth. Collectively these consequences would lead to less C supply to rhizobia in root nodules (Neo and Layzell, 1997). Similarly, the reduction in O_2 supply to root nodules under water stressed conditions could cause senescing of nodule tissues and bacterial cells (Marino et al., 2007). These plant physiological changes would lead to less N fixation and low grain yields (Guilioni et al., 2003) in many drought-prone fields. Therefore, there is a need to conduct more research on the success of these rhizobial inoculants under drought conditions in order to improve legume N nutrition.

1.3 The use of commercial rhizobial inoculants and their success against ineffective resident rhizobial populations in cropping fields

The presence of ineffective N fixing rhizobial populations is a major concern in Australian cropping soils (Siddique and Sykes, 1997), and effective rhizobial inoculation has proved an effective strategy to enhance N fixation of crop legumes (Howieson, 2016). Inoculation can

also increase the grain and biomass yield and post-crop soil nitrate levels (Van Kessel and Hartley, 2000). Inoculants are produced for different legumes in Australia (GRDC, 2013). Among 39 different inoculant groups produced commercially in Australia, group E (*Rhizobium leguminosarum* SU 303) and group F (*R. leguminosarum* WSM 1455) are commonly used for field pea inoculation (Drew et al., 2012). The use of various formulations and carrier materials assist in optimising the performance of commercial strains (e.g. peat based, freeze dried, granular, liquid forms) (GRDC, 2017). Peat-based inoculants provide reliable nodulation scores over the freeze-dried and liquid forms due to its protective formulation which avoids desiccation and pesticide exposure.

The optimal conditions for field pea inoculants are well-draining soils with a pH (water) of 6.0-7.5 (Rengasamy, 2016). The activity of commercial rhizobial inoculants is higher when resident rhizobial populations are small (Denton et al., 2002a). Further, most of the resident rhizobial populations are well-adapted to stressful soil conditions (Waldon et al., 1989). Some resident populations survive even under very low water potential (-1.0 MPa) in cropping soils (Busse and Bottomley, 1989) by increasing the average length of cells (e.g. 1.7 µm to 3.9 µm when stressed) in response to drought to enhance their infectivity with the host plant (Shoushtari and Pepper, 1985). Therefore, one major limitation for effective N fixation by inoculants in Australian fields is competition with resident rhizobial populations that are better adapted to prevailing stressful conditions (Peoples and Baldock, 2001). Given the extreme soil moisture conditions and competitive resident rhizobial populations in Australian soils, the success and persistence of a commercially produced rhizobial inoculant should be carefully monitored and managed. Studies evaluating the efficiency of different commercial rhizobial inoculants in a combination of biotic and abiotic constraints are lacking to date.

1.4 Importance of evaluating inter-strain interactions of rhizobia in order to enhance N fixation

Interactions among strains of rhizobia have been observed to affect the success of many legume symbioses (Trainer and Charles, 2006). One major ecological interaction existing between rhizobial strains is the competition for nodule space and host derived resources (carbon and oxygen) (Denison and Kiers, 2004a). The successful colonisation of a host by a rhizobial strain is also driven by several soil variables such as temperature, moisture content (Naylor and Coleman-Derr, 2018), acidity, heavy metal content (Hungria and Vargas, 2000) and some legume host variables such as root and shoot temperature (Gibson, 1967), root phenolics

(Vlassak et al., 1997) and defoliation (Gibson, 1971). Collectively, these factors could affect at a number of bacterial life stages and those can also influence interactions between rhizobia.

Assessing the genetic variation between rhizobial strains to evaluate inter-strain competitiveness is important in inoculant strain selection (Mutch and Young, 2004). Svanbäck and Bolnick (2006) proposed that the competition between individuals of the same species (intra-specific) could be low in a natural population if the individuals develop phenotypic variation. Further, it was explained that individuals having different phenotypes (detectable characteristics of an individual) may gain access to alternative resources (e.g. different carbon types) and would be able to avoid competition with more common phenotypes. Therefore, genetic similarity between rhizobial strains is considered a proxy for trait similarity in this study where genetic dissimilarity of rhizobial strains is expected to reflect differences in genetic traits such as in nodulation (*nod* genes) and acquisition of host carbon and oxygen. The purpose of relying on 16S rRNA is not to demonstrate that all traits are different among distantly related isolates, but to identify groups in which it is likely that functional variation exists including environmental interactions beyond host recognition and symbiotic establishment. For example, functional trait variation such as biofilm formation (Del Prado et al., 2012), utilisation of metabolise (Martiny et al., 2013), antibiotic resistance (Curt et al., 1984) can be considered beyond host recognition and N symbiosis. These genetic differences may reduce the intra-specific competition between rhizobial strains when colonising a host. If these distantly related rhizobial strains are also effective in N fixation, then the growth and yield of the host plant could potentially be promoted by inoculating multiple strains per root system. Therefore, this study reflects a novel approach of using rhizobial genetic similarity for evaluating the interactions between strains in multi-strain rhizobial inoculants to determine their synergistic effect on legume N fixation.

1.5 Effectiveness of multi-strain rhizobial inoculants versus single strain inoculants in legume N fixation

As described in section 1.2, one of the major biotic factors limiting rhizobial N symbiosis is the ineffective or reduced N fixing capacity of resident rhizobial populations in legume fields (Streeter, 1994). Therefore, the purpose of multi-strain rhizobial inoculants is to enhance the nitrogen fixation efficiency of the host plant by increasing the percentage of nodules with effective nitrogen fixing rhizobial strains. Multi-strain rhizobial inoculants are produced using

either rhizobial strains from two distinct species or strains from just one species of rhizobia (Roughley and Pulsford, 1982).

However, Somasegaran and Bohlool (1990) demonstrated the effectiveness of single-strain and multi-strain inoculants and the effect of soil N on the effectiveness and competitiveness of the strains. They observed that the amount of nitrogen fixed in a *Phaseolus vulgaris* (dry bean)-*R. leguminosarum* bv. *phaseoli* system with multiple strains was 51.8% lower than that of the single strain treatment of TAL 182 (the most effective treatment in the study). The main finding was that the strain that is the most effective at fixing nitrogen is not always the one that is most abundant in nodules when applied in combinations. Moreover, Brown and Ahmad (1996) provided more evidence for effects of inter-strain competitiveness on nodule occupancy in legume hosts. They examined the effectiveness of five *R. leguminosarum* bv. *phaseoli* strains (B2, B17, B36, T2 and CIAT652) in the *P. vulgaris* L. (Kidney bean) symbiosis. These strains were tested as single strains and as multiple strain inoculants and all the strains showed higher competitiveness (by occupying nodules ~ 64-79%) compared to resident rhizobia. When these strains were used in pairwise combinations, they did not always show an increase in nodule occupancy. For example, strain B17, which is competitive against resident populations on its own with ~68-70% nodule occupancy, showed lower nodule occupancy (~2.5%) when combined with the strain B2. They observed similar results with strain B17 when combined with B36 or CIAT652. However, from the practical and economic standpoint, formulation of multi-strain inoculants should consider the importance of selecting inoculants that are competitive as well as compatible with each other when applied in combinations. Therefore, current work has evaluated whether the multi-strain rhizobial inoculants were more efficient compared to single strain inoculants of *R. leguminosarum* in nodulation and N fixation of field pea.

Further, this study investigated an additional level of rhizobial competition in single nodule level. To date, there have been few studies focused on occurrence of two or more rhizobial strains in single nodules during multi-strain inoculations. For example, Moawad and Schmidt (1987) observed mixed nodule infections with multiple strains of *Bradyrhizobium japonicum* in early and late nodules of four soybean cultivars and a recent study by Checcucci et al. (2016) demonstrated that one fixing and another non-fixing strain of *Sinorhizobium meliloti* could occupy a single nodule in *Medicago sativa*. This study proposes that two *Rhizobium leguminosarum* strains share a single nodule if they are not competing with each other for space and host carbon/oxygen resources. It was hypothesised that the two genetically dissimilar

strains may have variation in nutrition acquisition traits such as different carbon nutritional patterns (Stowers, 1985 and Parke & Ornston, 1984). As mentioned in section 1.4, genetically distant *R. leguminosarum* strains may not have common phenotypes to acquire nutrients from the host and could co-occur in single nodules. Therefore, the synergistic interactions between these strains in single nodules may enhance N fixation in pea plants. Assessing nodule-level interactions of rhizobia in crop legumes provide better knowledge on selecting compatible pairs in multi-strain inoculant production.

1.6 Could root nodules be habitats only for rhizobia? Interactions between rhizobial strains and non-rhizobial endophytes in root nodules

Although rhizobia dominate the nodules of legume hosts, nodules can be occupied by other bacteria described as non-rhizobial endophytes (NREs) (Martínez-Hidalgo and Hirsch, 2017). Diverse populations of NREs have been observed in nodules and can affect N fixation directly or indirectly (Pandya et al., 2013). For example, some NREs (such as *Burkholderia* sp.) enhance legume nodulation and promote host growth when inoculated with rhizobia (Talbi et al., 2010, Soares et al., 2014). *Burkholderia cepacia* JBK9 has been studied for its ability as a bio-control agent by producing pyrrolnitrin against plant fungal pathogens including *Phytophthora capsici*, *Fusarium oxysporum*, and *Rhizoctonia solani* (Jung et al., 2018). Other NREs have also been studied but their effects on rhizobial function, if any, have not been determined (Reis and Teixeira, 2015).

Studies on the interactions between rhizobial symbionts and NRE are limited possibly due to the complexity in experimental design. It will also be important to investigate whether they compete with rhizobia for nodule occupancy in legumes. Would rhizobial symbionts provide sufficient niche space (the ecological position of a species in an ecosystem (Steele et al., 2011)) for NRE in host nodules? This study investigated whether there was a variation in NRE diversity and richness under single and multi-strain rhizobial inoculations in field pea. It was hypothesized that under single rhizobial strain inoculation, there might be less competition and more niche space for NREs in nodules. There is a lack of previous studies showing the interactions of rhizobial inoculation type and environmental constraints (such as drought) on the diversity and composition of these NRE in legume root nodules. To fill this knowledge gap, current work was also outlined to look at the composition and diversity of NRE found in field pea under drought conditions associated with different inoculations of *R. leguminosarum* strains. Further, specific NRE operational taxonomic units (OTUs) associated with drought and

rhizobial inoculations were identified for suggesting whether any beneficial NREs could be exploited for inoculant production to be applied in drought prone pea fields.

1.7 Research objectives

This project aimed to understand the effects of inter-strain interactions of *Rhizobium leguminosarum* on *Pisum sativum* L. (field pea) N symbiosis as rhizobium nitrogen fixation is an important phenomenon in field pea crop production in Australia (as mentioned in section 1.1, (GRDC, 2017, Poblaciones and Rengel, 2016)).

Therefore, this project focussed on the intra-specific interactions of rhizobial strains to investigate how they may influence efficiency of nitrogen fixation in field pea.

To address this, the following objectives are framed;

1. To determine how interactions among rhizobia affect competitive outcomes, and their consequences for nodulation and nitrogen fixation efficiency in field pea.

2. To determine how environmental extremes such as drought affect interactions among rhizobia and other bacteria, and consequences for N fixation.

This study proposed to provide new insights of inter-strain interactions of resident and inoculant rhizobia in legume symbiosis. Further the findings of this study will improve rhizobial symbiosis by adding more knowledge on multi-strain inoculant use and testing the efficiency of rhizobial strains under extreme environmental conditions such as drought. The emphasis on NRE composition and diversity among different rhizobial inoculation types would allow the industry to exploit plant growth promoting and N fixing abilities of NRE for inoculum production.

1.8 Thesis outline

Chapter 1: General Introduction

This chapter reviewed the ecological and economical importance of the rhizobium-legume symbiosis. The significance of inter-strain interactions of rhizobia on legume N fixation have been discussed emphasising the adverse effects of environmental extremes. This includes considerations of phenomena such as drought on rhizobial inter-strain interactions. The objective of this chapter was to introduce the knowledge gaps in our understanding of rhizobium-legume symbiosis regarding rhizobial competition, the effects of using commercial inocula and their persistence in legume fields. Further, the effects of ecological interactions between rhizobia on field pea N nutrition are described.

Chapter 2: Inoculation of field pea (*Pisum sativum* L.) with multiple *Rhizobium leguminosarum* strains improved root nodulation and nitrogen fixation

This chapter evaluated the inter-strain interactions of rhizobia by determining the success of inoculating field pea plants with multiple strains of rhizobia, compared with a single strain inoculation. Here, the following research questions were addressed;

1) Do inoculations with multiple strains of *Rhizobium leguminosarum* successfully enhance nodulation in *Pisum sativum* (field pea) plants when comparing with inoculation by a single strain?

2) If multiple strains of *R. leguminosarum* improve nodulation, does this treatment enhance nitrogen fixation and increase legume biomass, compared to single strain inoculation?

These questions were answered by conducting a pot experiment with field pea plants treated with different multi-strain combinations and single strain inoculations of *R. leguminosarum*. For analysis, the rhizobial inoculants were ranked by their genetic similarity. The plants inoculated with distantly related rhizobial pairs had significantly higher nodule numbers than plants with closely related pairs. Overall, multi-strain rhizobial combinations were more efficient than single strains in N fixation and nodulation although the effects on plant biomass were not significant. The results of this chapter led to the development of chapter 3, extending this research to better understand the efficiency of each individual strain and the effects of the interactions of rhizobial pairs at the single nodule level.

Chapter 3: Low frequency of infection by multiple *Rhizobium leguminosarum* strains in single nodules during rhizobial N symbiosis in field pea

To ascertain the prevalence of inter-strain competition of *R. leguminosarum* in single root nodules, the cohabitation of two strains of rhizobia in a single nodule was investigated. A plate-based plant growth experiment with different pairwise and single rhizobial strain treatments was established. A similar design to chapter 2 was applied here, with the strain combinations comprised of distantly and closely related pairs. My work showed that mixed nodule infections were infrequent in field pea-*R. leguminosarum* system. Nodulation and N fixation increased in plants inoculated with pairs of distantly related strains. However, interactions between distantly related pairs were not always cooperative, with some rhizobial strains outcompeted the other strain during nodulation. These competitive nodule forming strains did not always have high N fixation rates. Therefore, when selecting rhizobial strains for inoculation, individual strains

should be screened for their competitiveness in nodulation along with better N fixing ability to gain the symbiotic benefits.

Chapter 4: Drought and rhizobial competition reduced nodulation and N fixation by commercial *Rhizobium leguminosarum* in field pea

This chapter explored the effects of interactions between a commercial rhizobial inoculant (*Rhizobium leguminosarum* WSM1455) and competitor strains of *R. leguminosarum* on nodulation and N fixation in field pea plants. As well as testing the level of interaction between these strains, the negative affect of drought conditions on inter-strain interactions and field pea plant growth was monitored. This chapter also investigated whether there was an effect of the field pea cultivar on these inter-strain interactions by using two field pea varieties ('Twilight' and 'Wharton'). I conducted a pot experiments with field pea plants inoculated with WSM1455 and competitor strains (single and mixed strain inoculations), under well-watered and reduced watering treatment. WSM1455 alone produced bigger nodules with more N fixation. In contrast, N fixation, total N and plant biomass were significantly lower when WSM1455 was inoculated along with competitor strains suggesting that competition between WSM1455 and competitor strains might have reduced the efficiency of WSM1455. Alternatively, drought decreased N fixation and plant growth irrespective of the infected rhizobial strain. It is therefore important to select strains with competitive nodulation and N fixation when producing commercial inoculants. Current work has shown that the commercial inoculant was not successful under drought conditions which had low N fixation in pea plants. Therefore, to further understand these effects on traits under environmental stressors and develop more resistant rhizobial strains under field conditions, it would be necessary to conduct field-based testing.

Chapter 5: Drought and rhizobial inoculation altered communities of non-rhizobial endophytes in field pea nodules

To explore the drivers of rhizobial interactions with other endophyte bacteria in root nodules, this chapter investigated the diversity and composition of non-rhizobial endophyte (NRE) bacterial communities in field pea nodules under differing rhizobial and watering conditions. It was hypothesized that rhizobial competition and drought could alter the diversity of NRE in nodules. The diversity and composition of NRE was calculated from *Rhizobium leguminosarum* root nodules collected during the experiment described in chapter 4. I then analysed the effects of differing watering and rhizobial treatments on NRE diversity and composition. Under

drought, the diversity of NRE increased in mixed rhizobial inoculation (WSM1455 + competitors) but decreased in single inoculations treatments. There were distinct NRE communities associated with competitor rhizobial inoculation in reduced and well-watered treatments. Overall, the results of this chapter demonstrated the importance of identifying and exploiting the beneficial NRE bacterial species in the future, in order to be incorporated in rhizobial inoculant production.

Chapter 6: General Discussion

This chapter summarised the key findings of this project and how my data addressed the hypotheses proposed in the study. Further, chapter 6 described the novel aspects of rhizobial N fixation to be developed in future work using the valuable outcomes of the current study.

Chapter 2: Inoculation of field pea (*Pisum sativum* L.) with multiple *Rhizobium leguminosarum* strains improved root nodulation and nitrogen fixation

2.1 Introduction

Nitrogen (N) is one of the essential nutrients for plant growth (Verhoeven et al., 1996) but activities related to agricultural practices including tillage, biomass removal and leaching (Vitousek et al., 2002) result in loss of N from cropping soils. Legumes establish a symbiotic interaction with N fixing bacteria commonly called rhizobia (Oldroyd et al., 2011). Atmospheric nitrogen (N_2) is converted enzymatically to ammonia/ ammonium (NH_3/NH_4^+) by rhizobial bacteria within a specialized root structure called a 'nodule'. The rhizobial-legume symbiosis involves bidirectional signal exchange mechanisms in an exclusive plant-bacterial relationship (Trainer and Charles, 2006). Rhizobia are capable of infecting agriculturally important legume crops including chick pea (*Cicer arietinum*), cow pea (*Vigna unguiculata*), faba bean (*Vicia faba)* , dry bean (*Phaseolus* sp.), French bean (*Phaseolus vulgaris*), lupin (*Lupinus* sp.), lentil (*Lens culinaris*) , soy bean (*Glycine max*), alfalfa (*Medicago sativa*), pea (*Pisum sativum*), ground nut (*Arachis hypogaea*) and field pea (*Pisum sativum*) , with these legumes covering more than 220 million ha of the world's cropping land by 2014 (Stagnari et al., 2017). The worldwide contribution of rhizobial nitrogen fixation in agricultural crop production is estimated to be 21 million tonnes of N per year (Herridge et al., 2008). Field pea is one of the most widely- grown legume crops and has an annual production of 0.3 million tonnes in Australia (Poblaciones and Rengel, 2016).

Better establishment in the rhizosphere environment and building-up root symbiotic associations are key processes in the rhizobial life cycle (Ambrosini et al., 2016). Further, the presence of non-rhizobial nodule endophytes (NREs) such as *Pseudomonas* sp., *Paenibacillus* sp., *Acidovorax* sp. and *Chryseobacterium* sp., which can occupy root nodules with rhizobial strains, has been reported in many studies (Philipson and Blair, 1957, Sturz et al., 1997). It has observed that mixed inoculations with rhizobia and other endophytic bacteria such as *Bacillus* sp. promote root nodulation and enhances N fixation (Schwartz et al., 2013). These non-rhizobial endophyte (NRE) communities can vary from being mutualists or commensals to parasites in terms of their effect on the host plant (Sturz and Nowak, 2000). It is not known whether these communities interact directly and/or indirectly in legume symbiosis or have no effect at all.

There can be significant variation in the abundance and diversity of rhizobial populations in cropping soils (Carelli et al., 2000). Further, Thies et al. (1992) suggested that soil fertility is one of the most important factors in nodule occupancy of rhizobium symbionts. Among the large array of environmental variables affecting symbiotic N fixation (such as pH, drought stress and soil type), the effects of the competitive inter-strain interactions of resident rhizobia for limiting resources can be considered as an important biotic factor (Peoples et al., 2012). It has been suggested that some resident soil rhizobial strains are ineffective in fixing Nin host plants despite being successful colonizers of plant roots (Thrall et al., 2000). Further, Ampe et al. (2003) found that there are specific genes involved in rhizobial competitiveness. Under field conditions, the ineffective resident rhizobial strains can be highly competitive host plant colonizers causing significant reduction in crop yield (Triplett and Sadowsky, 1992). Therefore, inoculation of legumes/ seeds at early seedling development with more effective N fixing rhizobial strains is vital in most of the legume crop fields (Drew et al., 2012) where they have to withstand the competitive pressure of resident strains.

Increased yield has been observed under *Medicago sativa* (alfalfa) inoculated with pairs of *Rhizobium meliloti* demonstrating synergistic interactions between rhizobial strains and could be further improved to enhance the yield of legume crops and overcome competitive pressure of resident strains (Bromfield, 1984). However, it has also been shown that antagonistic effects of mixtures of rhizobial strains can also cause poor crop yields (Heath and Tiffin, 2007). Collectively, these studies imply the need for the evaluation of inter-strain compatibility of rhizobia in enhancing N fixation efficiency in legume crops. Some legume hosts prefer colonization of multiple strains of rhizobia per root system (Somasegaran and Bohlool, 1990). Here, I hypothesized that field pea hosts favor the compatible co-inoculations of *R. leguminosarum* strains to gain more N benefits. The genetic diversity of rhizobium inoculants could be used for selecting better N-fixing rhizobial partners (Mutch and Young, 2004) since rhizobia are known to have a high degree of genetic diversity and host specificity in nature (Simms and Taylor, 2002). Genetically dissimilar rhizobial strains would have differences in nodulation (*nod*) and N-fixation (*nif*) genes leading host plants to select more effective N fixers during the nodulation process (Checcucci et al., 2016).

Further, the synergistic behavior of rhizobia can be affected by the inter-strain competition of closely related rhizobia in root nodules (Kiers and Denison, 2008). Therefore, I suggested that the compatibility of a rhizobial pair would depend on the degree of genetic similarity between each other where genetically more similar strains in a rhizobial pair would be less compatible, competing strongly with each other. Closely related rhizobial strains can hinder the synergistic

N fixation capacity due to their competition for host resources in the root nodule (Platt and Bever, 2009). I also suggested that the strong inter-strain competition in highly similar rhizobial combinations will result less contribution to N fixation compared to when inoculated singly. The current study looked at the inter-strain interactions of *Rhizobium leguminosarum* in *Pisum sativum* (field pea) symbiotic system. In particular, I aimed to address the following questions:
I) Does the symbiotic interaction of *Rhizobium leguminosarum – Pisum sativum* allow successful nodulation of rhizobial co-inoculations over single strain inoculations?
II) If co-inoculations are successful then are these rhizobial co-inoculations efficient in nitrogen fixation with field pea biomass increase compared to single strain inoculations?
Having learnt that strain-level variants within a bacterial species can be diverse in functional traits and interactions with host tissues (Bron et al., 2012), my study system used the genetic similarity of strains of *R. leguminosarum* as a proxy for evaluating the compatibility of rhizobial pairs in enhancing field pea N symbiosis. I have used two soil types in this experiment (red calcarosol and black vertosol) to test whether the responses of different rhizobial co-inoculations in field pea N symbiosis are generalizable.

2.2 Materials and methods

2.2.1 Selection of strains used in this study

A total of twelve *Rhizobium leguminosarum* strains (obtained from the Department of Jobs, Precincts, and Regions, Agriculture Victoria Research Division, culture collection AgriBio Centre, VIC, Australia) were used to construct a tree of phylogenetic similarity. The original collection details of these strains such as associated crops, and geographic locations can be found in Table S2-1, Appendix 1. The isolated colonies from rhizobial cultures were lysed using 3% KOH, heating at 95°C for 2 min followed by cooling on ice and centrifuging (Eppendorf miniSpin plus, 22331 Hamburg, USA) at 8000g for 2 min (Rouhrazi and Rahimian, 2012). The supernatant was taken as template for colony PCR using ERIC primers (Wilson and Sharp, 2006). The approach of 16S *rRNA* sequences has been used previously by others to differentiate strains according to their genetic similarity (e.g., Johnson et al., 2019 and Morey et al., 2006). 16S rRNA sequences were generated from DNA of these isolates after colony PCR amplification with 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Suzuki and Giovannoni, 1996) primers and PCR conditions are initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 secs, primer annealing at 55°C for 30 secs, extension at 72°C for 90 sec and final extension at 72°C for 6

min (Eichorst et al., 2011). The amplified PCR products were sequenced on Sanger sequencing facility at Hawkesbury Institute for the Environment (HIE), Western Sydney University (Hitachi Applied BioSystems 3500 Genetic Analyzer 8ch RUO 622-0010, Hitachi High Technologies Corporation, Tokyo, Japan) using 518F (5'-CCAGCAGCCGCGGTAATACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3') (Lamsal et al., 2012) as sequencing primers. The strain sequences had average length of ~1400bp. It was suggested that the sequence length of ~ 1400bp could reflect strain-level variation (Johnson et al., 2019). Their genetic similarity varied from 97 to 99.5% across all pairs of isolates. The phylogenetic tree was constructed in MEGA version 7.0 using the 16S rDNA sequences (obtained from SANGER sequencing facility at HIE) of 12 *Rhizobium leguminosarum* strains. The sequences were aligned using the program Multiple sequence Alignment (MAFFT) version 7.0 (Katoh and Standley, 2013) and the resulting alignments were fed to MEGA 7.0 with 1000 bootstrap replications to obtain the maximum likelihood tree. Five strains were selected out of 12 which could be separated via Enterobacterial Repetitive Intergenic Consensus (ERIC) fingerprinting (Leung et al., 2004) using unique banding patterns of these strains (See chapter 3, section 3.2.1 for more details of ERIC primers and ERIC PCR conditions). Using the five *R. leguminosarum* strains, seven pairs were designed according to their genetic similarity (see Section 2.3- Results, Figure 2.1 and Table 2.1).

2.2.2 Seed germination, planting and inoculation

Pisum sativum (field pea) cultivar Yarrum was used in this experiment. Seeds were surface-sterilized with 0.5% NaOCl for 1 min followed by six washes of milli-Q water (Somasegaran and Hoben, 1985). Seeds were germinated in vermiculite and the two-week-old germinated seedlings were transferred to pots (W:65 mm, H:150 mm, L:65 mm) filled with one of two soils collected from paddocks in western Victoria, Australia: a vertosol ('black', Wimmera region, organic carbon content (1.43%) and NH_4^+ (6.33 mg/kg soil)) and a calcarosol ('red', Mallee region, 0.47% organic carbon and <1 mg/kg NH_4^+) each mixed with an equal (1:1) volume of vermiculite. The soils were twice autoclaved at 121°C for 1 hour before use to get rid of any soil rhizobia. Plants were watered with 20-30 ml of sterile (autoclaved at 121°C) milli-Q water. All the pots were fertilized with 25 ml of N-free nutrient solution (Somasegaran and Hoben, 1985) (Table S2-2, Appendix 1) Further, the pots were well-spaced in the growth chamber and vermiculite was used in pots to avoid cross contamination.

For inoculum production, selected RRI isolates (Table 2.1) were cultured in 20 ml of Yeast Mannitol Broth (YMB) and incubated on a rotary shaker (RATEK, ROWE Scientific Pty Ltd)

at 25°C and 130 rpm. Culture density was assessed spectrophotometrically at 600 nm wavelength (Plate Reader G6860A, Agilent Technologies, Cary 60 UV-Vis) to standardise the concentration of inoculum to 10^7 cells/ml added to each pot. On the same day, single strains and strain combinations (Table 2.1) were inoculated to relevant pots (four replicates per treatment). Each pot contained either of soils up to 1 cm below the rim. Inoculated plants were watered (20 ml/plant, free draining) once in four days and fertilized with N-free nutrient solution (25 ml/plant) once a week until the harvest. The plants were grown for six weeks under ambient temperature (26°C daytime and 18°C night) in a glass house before harvesting. The total of 108 field pea plants were grown belonging to two soils, treated with seven rhizobial combinations, five single strain inoculations and uninoculated controls (each treatment had four replicate plants).

2.2.3 Harvesting and collection of nodules, shoots and roots for analyses

Plants were harvested at six weeks when they were well-nodulated. Plants were carefully removed from the soil and roots were washed gently with running water to remove soil and vermiculite. The washed plants were kept on clean, moist tissue papers and the total number of nodules were counted. Out of 22 uninoculated plants grown in black, red and vermiculite substrates, only 2 plants showed few nodules (~4-5) which was considered as minor cross contamination. Nodule samples from dual inoculated plants were placed in 96 well plates (5 from upper root system, 5 from mid-root system and 5 from lower root system) and frozen at -20°C for DNA extractions for strain identification. Shoot and root parts were separately placed in paper bags, dried at 70°C for 72 hours and dry weight was determined. Shoot tissue was analysed for total N and ^{15}N (natural abundance) concentrations at the West Australian Biogeochemistry Centre (Crawley, WA, Australia) on an Isotope Ratio Mass Spectrometer with EA (Delta V^{TM}, Thermo Fisher Scientific).

The percentage of nitrogen derived from the atmosphere (%Ndfa) was determined for shoot samples (each soil) using the following formula (Polania et al., 2016).

$$\%Ndfa = \frac{\delta^{15}N \text{ of the reference plant} - \delta^{15}N \text{ of test legume}}{\delta^{15}N \text{ reference plant} - \beta} \times 100$$

Reference plant= un-inoculated pea plants (in each soil)

(Note: The reference plant was not inoculated with rhizobia to confirm there is no rhizobial N fixation.

$\beta = \delta^{15}N$ of the nodulated pea plant solely dependent on fixed N (grown in vermiculite) for N requirement).

$$\text{Fixed amounts of N (mg)} = \frac{\text{Total Amount of N (mg) in shoot sample*} \times \%\text{Ndfa}}{100}$$

*Total amount of N was analysed along with the ^{15}N concentrations at WA

2.2.5 Analysis of root nodules for the presence/identification of rhizobia

The nodules stored (in -20°C freezer) after harvesting were taken for rhizobial isolations. The nodules were sterilized with 0.5% NaOCl for 1 minute and followed by six washes of sterile milli-Q water (. Attempts to identify strains at the nodule level were not successful. Therefore, 16 composite nodule samples were made where five nodules from each of four replicates of the same treatment were combined (20 in total). Resulting DNA fragments were amplified using 27F and 1492R primers (Eichorst et al., 2011). PCR products were purified using Agencourt® AMPure PCR purification kit and DNA concentrations were measured using Nanodrop (NanoDrop 2000c spectrophotometer, Thermo Fisher Scientific). Luria-Bertani (LB) medium was prepared using 35 g of LB agar powder dissolved in 1 L milli-Q water which was adjusted at pH 7.0 with sodium hydroxide and sterilized by autoclaving (Tanabe et al., 2002). LB plates were made with LB medium mixed with ampicillin 100µg/ml. Cloning reaction was performed using TOPO® TA Cloning® Kit (K4500J10-Invitrogen). Spread plates were prepared using two volumes 50 µl and 100 µl of each cloned and transformed sample. Fifteen positive clones (white colonies) from each sample were picked with sterile needles and lysed using thermocycler (Dyad Peltier Thermal Cycler) at 94°C for 10 min. PCR was performed for these lysed colonies using the T7 primer 5´-TAATACGACTCACTATAGGG-3´ (Tabor and Richardson, 1981) with either 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers (Suzuki and Giovannoni, 1996). Approximately 10 PCR products were selected per each sample for purification and sequenced using 518F (5'-CCAGCAGCCGCGGTAATACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3') sequence primers (Lamsal et al., 2012) at Sanger sequencing facility at HIE.

2.2.6 Data Analysis

All statistical analyses used R version 3.3.1 (R Development Core Team, 2016). For each plant response, averages of four replicates in each treatment were used because our experimental

units were the combinations of strains, not the individual plants. Data were analysed in two ways: (1) linear models were fitted to average responses from all treatments inoculated with two strains and (2) linear models were fitted to ratios of combination versus single inoculation responses for each strain found in a combination (log response ratios) (Yuan and Chen, 2015).

Response ratio = ln (response [combination]/response [single strain])

All the models were examined with diagnostic plots (QQ-plots and scale location plots) of residuals to check whether the model fitted the data well (the fitted models were used in the analyses). Two-way ANOVA was performed to determine the effects of rhizobial genetic similarity and soil type on each of the plant responses measured in co-inoculation treatments (i.e., nodule number, fixed N amounts, total N amounts, shoot: root and total plant biomass). To determine whether the low genetic similarity group was associated with significantly increased plant response ratios compared to high similarity groups, *post hoc* analyses of multiple comparisons using the R package 'emmeans' to calculate Tukey's Honestly Significant Difference test. I compared the means of High versus Low genetic similarity under each soil type used in the experiment. I have done two-sample t-tests comparing the ratios of plant response variables in red calcarosol and black vertosol soils where the rhizobial genetic similarity had less significant effects. The figures were constructed using the package 'ggplot2' (Wickham, 2009) in R 3.5.1 using the co-inoculated plant response variables and their response ratios observed under each rhizobial genetic similarity group and soil type.

Following the sequencing of nodule bacterial DNA, the resulting forward and reverse DNA sequences were aligned and trimmed using Geneious 10.2.3 and contigs were annotated taxonomically using NCBI Nucleotide BLAST (Altschul et al., 1990). Using the BLAST results rhizobial sequences were aligned with RRI strain sequences using the program Multiple sequence Alignment (MAFFT) version 7.0 (Katoh and Standley, 2013) to identify the strain ID in each nodule sample. The effect of rhizobial genetic similarity on the colonisation of either rhizobial or non-rhizobial strain in nodules was analysed using generalized linear modelling (GLM- binomial distribution in R, Analysis of Deviance Table (Type II tests)).

2.3 Results

2.3.1 Analysis of the genetic similarity between *R. leguminosarum* strains

The phylogenetic tree in Figure 2.1 shows the genetic similarity of the twelve *Rhizobium leguminosarum* strains used in this study. Table 2.1 shows the strain combinations based on their genetic similarity.

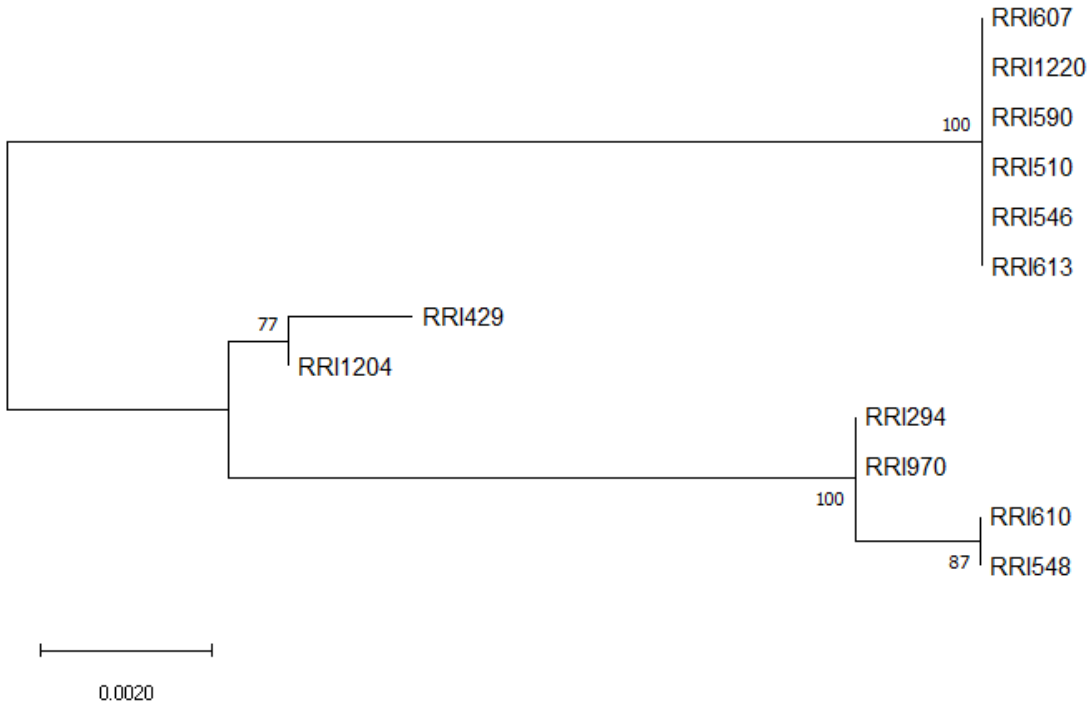

Figure 2. 1: Maximum likelihood tree for 16S rDNA sequences of Resident Rhizobial (RRI) strains using HKY (Hasegawa-Kishino-Yano) model with 1000 bootstrap replications. The tree was built in Molecular Evolutionary Genetics Analysis Version 7.0 (MEGA7). The numbers indicated in tree nodes shows the bootstrap support for the branching split in each case. The scale bar indicates the proportion of bases changing along the branches.

The selected strains of *R. leguminosarum* varied in their genetic similarity (Figure 2.1) with six highly similar RRI strains at 100% bootstrap support (BS) and the remaining six strains diverged into separate clusters at BS 87% (RRI610 and RRI548), BS 77% (RRI429 and RRI1204) and BS 100% (RRI294 and RRI970). Pair-wise comparisons using NCBI BLAST showed that RRI429, RRI294, RRI548 and RRI970 had 99% similarity (E=0.0, Query cover=99%). Five strains were selected (see Table 2.1) using unique ERIC banding patterns to make combinations (Figure S2-1, Appendix 1).

Table 2. 1: *Rhizobium leguminosarum* strain combinations based on their genetic similarities

Low similarity	High similarity
RRI 1220/ RRI 294	RRI 548/RRI 970
RRI 1220/RRI 429	RRI 970/RRI 429
RRI 1220/RRI 970	RRI 294/RRI 429
	RRI 294/RRI 970

2.3.2 The effects of rhizobial genetic similarity on measured plant response variables

I analyzed the effects of genetic similarity of *R. leguminosarum* pairs on field pea plant response variables (i.e. Nodule number, Fixed amount of N, Total N and Plant biomass). Further, the strain combination responses were compared with single strain responses (co-inoculation effect ratios) to determine the degree of efficiency of a rhizobial combination to enhance plant growth and N fixation.

2.3.2.1 Field pea plants with genetically less similar rhizobial pairs had more root nodules

Nodule number increased by 75% and 66% in black vertosol and red calcarosol soils respectively when co-inoculated with genetically dissimilar ('low' similarity) *R. leguminosarum* strains compared to the plants co-inoculated with genetically similar ('high' similarity) strains ($P_{\text{similarity}}$ = 0.06, Two-way ANOVA, Table 2.2, Figure 2.2).

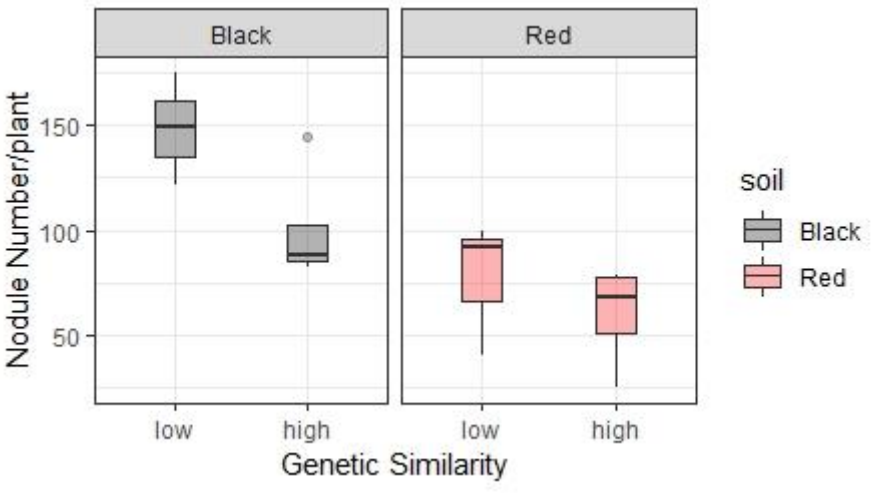

Figure 2. 2: Boxplots indicating the number of nodules per plant root for pea plants co-inoculated with pairs of *Rhizobium leguminosarum* strains differing in their degree of genetic similarity (based on 16S rDNA sequences). Responses were measured for plants grown in two different soils: a black vertosol ('black') and a red calcarosol ('red'). Results shown are for n=4 (biological triplicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles. The vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range. $P_{\text{genetic similarity}}$ = 0.06, $P_{\text{soil}} < 0.01$, $P_{\text{interaction}}$ = 0.34 (Two-way ANOVA).

There were significant differences in root nodulation ($P<0.05$, Two-way ANOVA) between two soil types used in the study. The plants grown in black vertosol soil had an average of ~120 nodules/plant whereas the plants in red calcarosol had ~60 nodules/plant on average regardless of the rhizobial similarity group. The interactive effect of rhizobial genetic similarity and soil type on the observed nodule numbers of pea hosts was not significant ($P=0.34$, Two-way ANOVA, Table 2.2). The nodule numbers recorded for the genetically less similar group in the black vertosol soil was 50% higher compared to the high similarity group ([$\text{Black}_{\text{low}}$ vs. $\text{Black}_{\text{high}}$], $P= 0.05$, multiple comparisons ('emmeans'). There were no significant differences in nodule numbers between the genetic similarity groups in red calcarosol soil.

2.3.2.2 No sufficient evidence that rhizobial genetic similarity affects the N symbiosis in host plants

My data did not support the hypothesis that the genetic similarity of rhizobia affects the fixed and total amount of assimilated N in field pea hosts ($P=0.07$, Two-way ANOVA, Table 2.2, Figure 2.3). Pair-wise comparisons between rhizobial similarity groups for N responses showed no significant differences in both soil types ($P>0.05$, [$\text{FixedN}_{\text{high similarity}}$ vs. $\text{FixedN}_{\text{low similarity}}$] and [$\text{TotN}_{\text{high similarity}}$ vs. $\text{TotN}_{\text{low similarity}}$], multiple comparisons ('emmeans')).

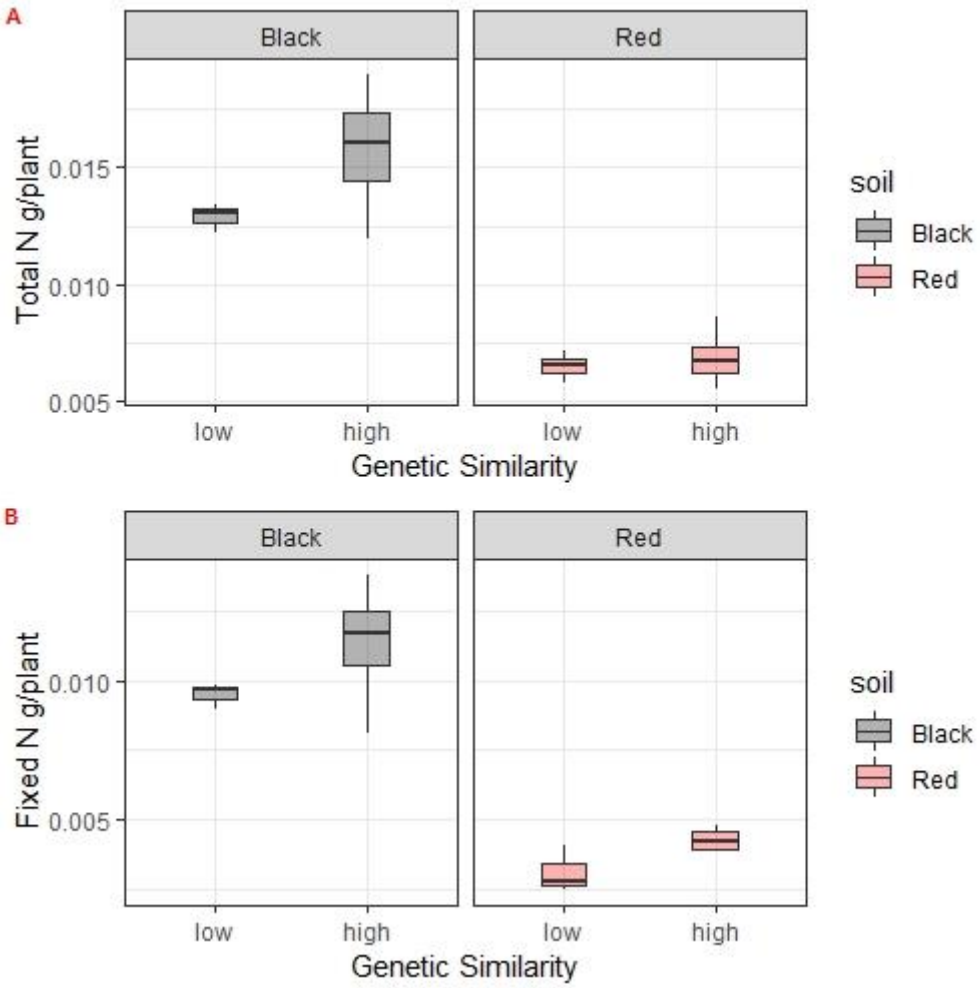

Figure 2. 3: Boxplots representing A. Amount of total nitrogen (g) per plant B. Amount of atmospherically fixed nitrogen (g) per plant co-inoculated with pairs of *Rhizobium leguminosarum* strains grouping according to their genetic similarity. Effect of soil for N responses was significant at $P<0.05$ (calculated from two-way ANOVA) where the effect of genetic similarity and the interaction between soil and genetic similarity were not significant ($P>0.1$) for the above responses (A, B) measured. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles. The vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range.

Further, the total nitrogen uptake by the plant and rhizobial N fixation were greater (one-fold increase) for plants growing in the black vertosol than those in the red calcarosol ($P<0.01$, multiple comparisons [$TotN_{\text{black soil}}$ vs. $TotN_{\text{red soil}}$] and [$FixedN_{\text{black soil}}$ vs. $FixedN_{\text{red soil}}$]). This work lacked the evidence for a significant interaction between genetic similarity and soil type affecting plant N benefits ($P_{\text{interaction}} > 0.1$, Two-way ANOVA, Table 2.2, Figure 2.3).

2.3.2.3 Rhizobial genetic similarity was not a significant predictor for field pea biomass responses

Genetic similarity was not a significant predictor of shoot and root biomass or total plant biomass responses measured in the experiment ($P_{\text{similarity}}>0.05$, Two-way ANOVA, Table 2.2,

Figure 2.4). The soil order had significant effects on shoot and root biomass (P_{soil}<0.01, Table 2.2) with plants grown in black vertosol having more biomass (1-fold increase) compared to the plants grown in red calcarosol irrespective of the genetic similarity of rhizobial infection type (P<0.05, [Biomass $_{black}$ vs. Biomass $_{red}$], multiple comparisons ('emmeans'), Figure 2.4)

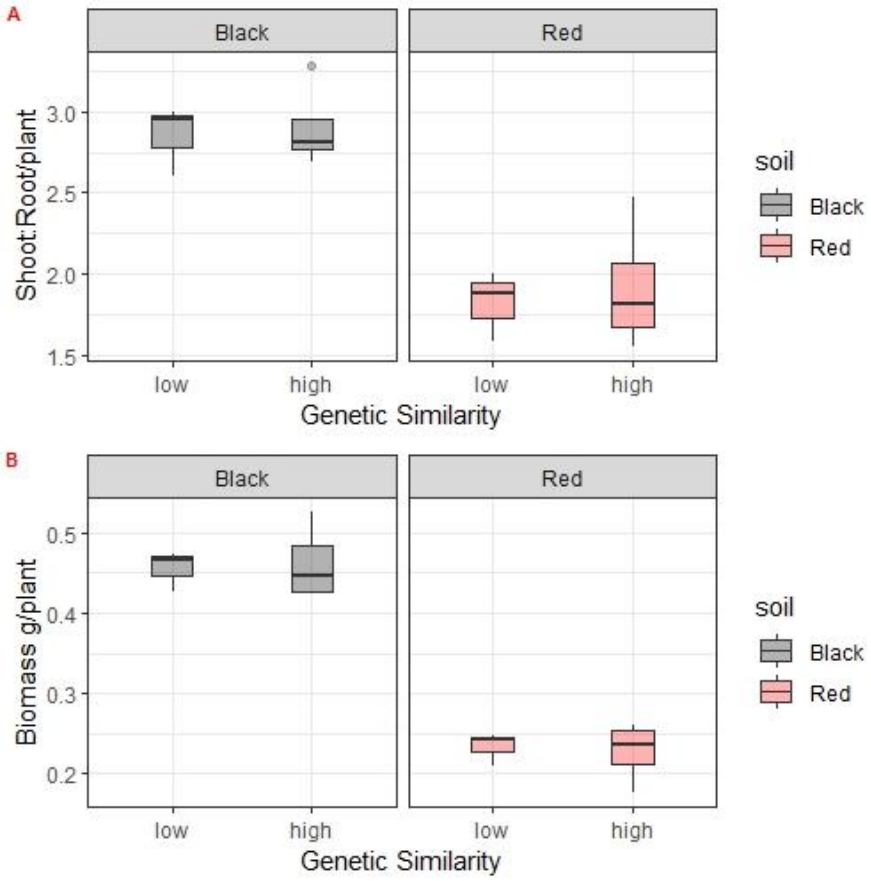

Figure 2. 4: Boxplots indicating A. Shoot: Root biomass ratio per plant B. Total biomass of the plant (g) where the pea plants were co-inoculated with combinations of *R. leguminosarum* strains (n=4) according to their genetic similarity. Values indicated are per plant basis. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles. The vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range. Effects of soil for both the responses were significant at P<0.05 (calculated from two-way ANOVA and multiple comparisons between two soil types using 'emmeans') where the effects of genetic similarity and interaction of soil and genetic similarity were not significant.

The interactive effect of the genetic similarity of rhizobia and the soil order had no significant role on plant biomass responses (P>0.05, Two-way ANOVA, Table 2.2).

Table 2. 2: Summary of two-way ANOVA showing the effects of rhizobial genetic similarity and the soil type on the nodule number, % Nitrogen derived from atmosphere (%Ndfa), amount of fixed nitrogen, Total nitrogen content, shoot, root and total biomass of plants inoculated with rhizobial pairs

Effect	**Degrees of Freedom**	**Nodule number**		**% Ndfa**		**Fixed N**		**Total N**		**Shoot biomass**		**Root biomass**		**Total plant biomass**	
		F	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Genetic Similarity	1,10	4.54	0.058†	1.03	0.33	3.96	0.07†	2.71	0.13	0.06	0.8	0.97	0.34	0	0.99
Soil	1,10	12.57	<0.01 **	3.6	0.09†	80.5	<0.01 **	64.86	<0.01 **	118.3	<0.01 **	77.06	<0.01 **	134.8	<0.01 **
Genetic similarity*Soil	1,10	0.98	0.343	1.54	0.24	0.17	0.68	1.6	0.23	0.06	0.81	0.16	0.69	0.095	0.76

Note: '**'for *P*<0.01,'*' for *P*<0.05 and '†' for *P*<0.1

2.3.2.4 The co-inoculation effect of rhizobia on plant nodule number did not significantly vary with their genetic similarity or the type of soil

The co-inoculation response of rhizobia showed a marginal increase (~12% in black and ~40% in red soils) in nodule number with the low genetic similarity group compared to the high similarity group (P=0.06, Two-way ANOVA, Table 2.3, Figure 2.5). However, there were no significant interactive effects for soil and genetic similarity on the response ratios of nodulation (P>0.05, Table 2.3).

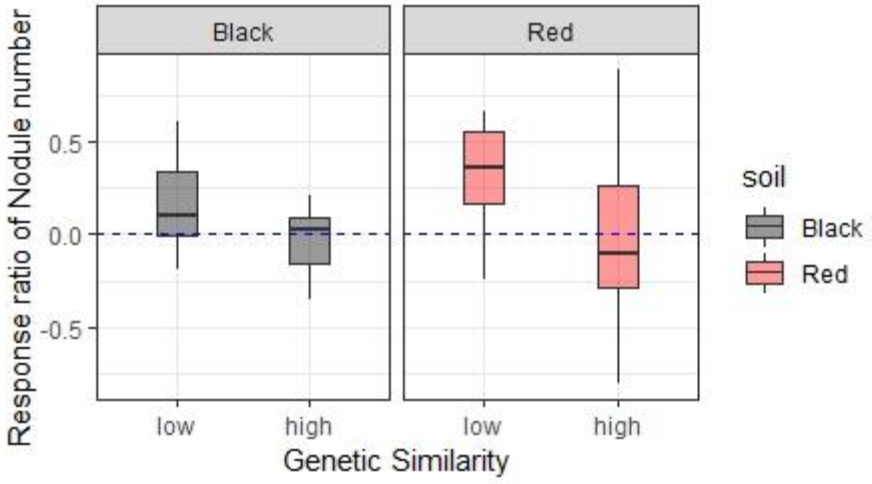

Figure 2. 5: Effect of co-inoculation on the nodulation response by a single rhizobial strain in a combination. The zero line depicts no net co-inoculation effect for response variable when the isolate is combined with another isolate. The positive response above the zero line indicates increased response of co-inoculation with pairs compared to on its own performance of strains and negative responses show that the performance of a strain on its own more effective than when co-inoculated with another strain. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles. The vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range. The effect of genetic similarity for relative nodulation response was marginally significant ($P_{similarity}$ = 0.06, P_{soil} =0.62, $P_{interaction}$ = 0.64, tested with Two-way ANOVA). Nodulation response values of 'low' genetic similarity group lie above the zero-line regardless to which soil type they belong.

Moreover, the effect of soil was not significant on the response ratios of plant nodule number (P>0.05, [Nod $_{black}$ vs. Nod $_{red}$], Multiple comparisons ('emmeans')).

2.3.2.5 The genetic similarity of rhizobia did not significantly affect the co-inoculation response of fixed and total amounts of nitrogen in pea plants

The co-inoculation response for fixed and total amounts of nitrogen in pea plants (Figure 2.6 (A) and (B)) did not differ significantly among genetically low and high similarity groups of rhizobial strains used in the study (P>0.05, Two-way ANOVA, Table 2.5).

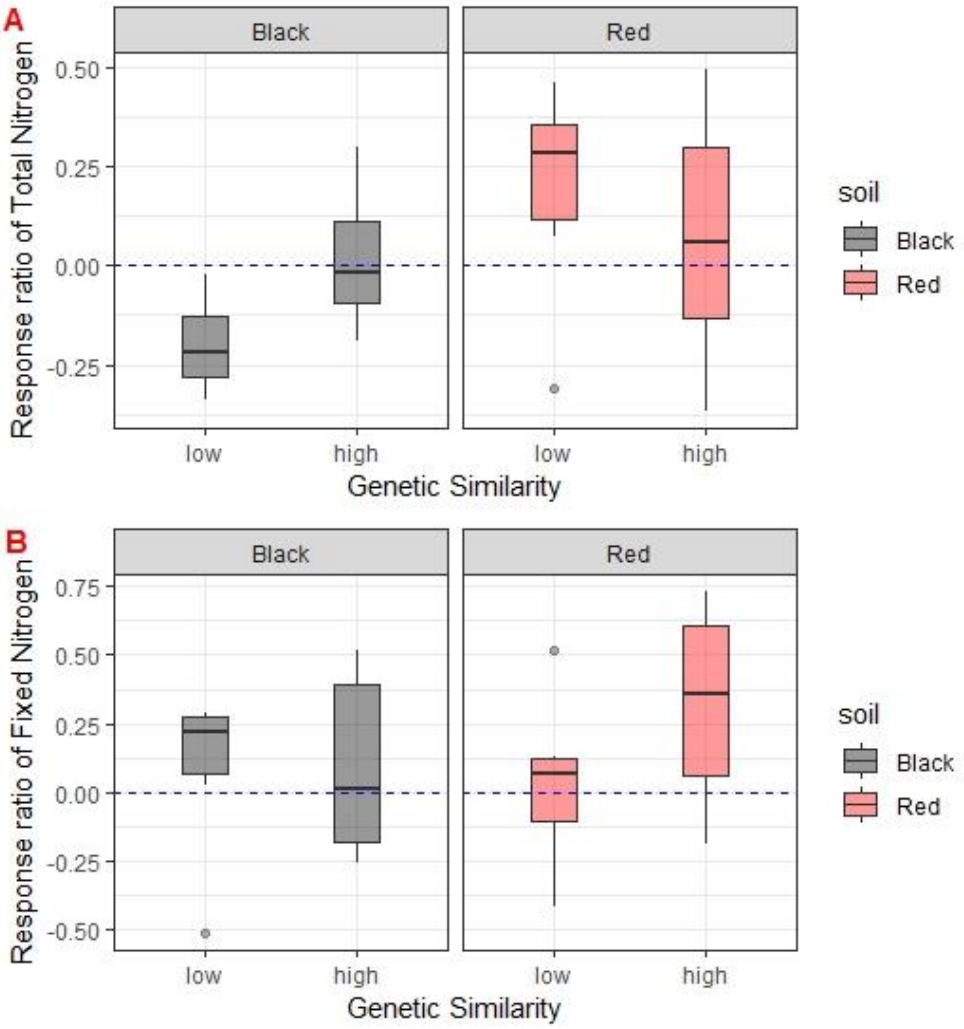

Figure 2. 6: Net co-inoculation effect on the (A) total nitrogen content of the plant and (B) total amount of fixed nitrogen per plant by rhizobial combination compared to a single isolate in the combination. The zero line depicts there is no net co-inoculation effect for response variable when the isolate is combined with another isolate. The positive response above the zero line indicates increased response of co-inoculation with pairs compared to on its own performance of strains and negative responses show that the performance of a strain on its own more effective than when co-inoculated with another strain. The effect of genetic similarity for total nitrogen and fixed nitrogen is at P>0.05 whereas the soil type shows significant effect at P=0.04 and $P_{interaction}$ = 0.07, tested with Two-way ANOVA. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles. The vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range.

There was a significant interactive effect between rhizobial similarity and soil type on the net co-inoculation effect when total amount of N assimilated in pea plants was measured (P=0.04, Two-

way ANOVA, Table 2.3). The total N response of the plants inoculated with genetically low rhizobial similarity group in black soil was approximately ~25% less than the amounts of total N plants inoculated with the high rhizobial similarity group. In contrast, the genetically low rhizobial similarity group in red soil coincided with 25% higher total N for co-inoculation effect compared to high similarity group in red soil. Due to the variation in N data observed, there were no significant differences between the rhizobial similarity groups within each of the soil types (P>0.05, [$TotalN_{\text{low similarity.red soil}}$ vs. $TotalN_{\text{high similarity.red soil}}$] and [$TotalN_{\text{low similarity.black soil}}$ vs. $ToatlN_{\text{high similarity.black soil}}$], Multiple comparisons ('emmeans')).

In general, my work provided the evidence that the co-inoculation effect on total N is significantly increased (~100%) in field pea plants grown in red calcarosol soil compared to black vertosol soil (P=0.04, Two-sample t-test, Table 2.4).

2.3.2.6 A significant interaction between rhizobial genetic similarity and soil type affected the co-inoculation response of total plant biomass

Rhizobial genetic similarity had less significant effects on co-inoculation responses when expressed as plant biomass (P>0.05, Two-way ANOVA, Table 2.3). A significant interactive effect of rhizobial genetic similarity and the type of soil on the co-inoculation response of shoot mass and total plant biomass response ratios was observed (P=0.04 and P=0.05 respectively, Two-way ANOVA, Table 2.3). Total plant biomass and shoot mass responses of genetically low similarity group in black soil showed more negative values (~12% and ~13% less respectively) compared to high similarity group. In contrast, genetically low similarity rhizobial group in red soil had ~15% and ~20% higher total biomass and shoot mass responses for co-inoculation effect compared to high similarity group (Figure 2.7(A) and (B)).

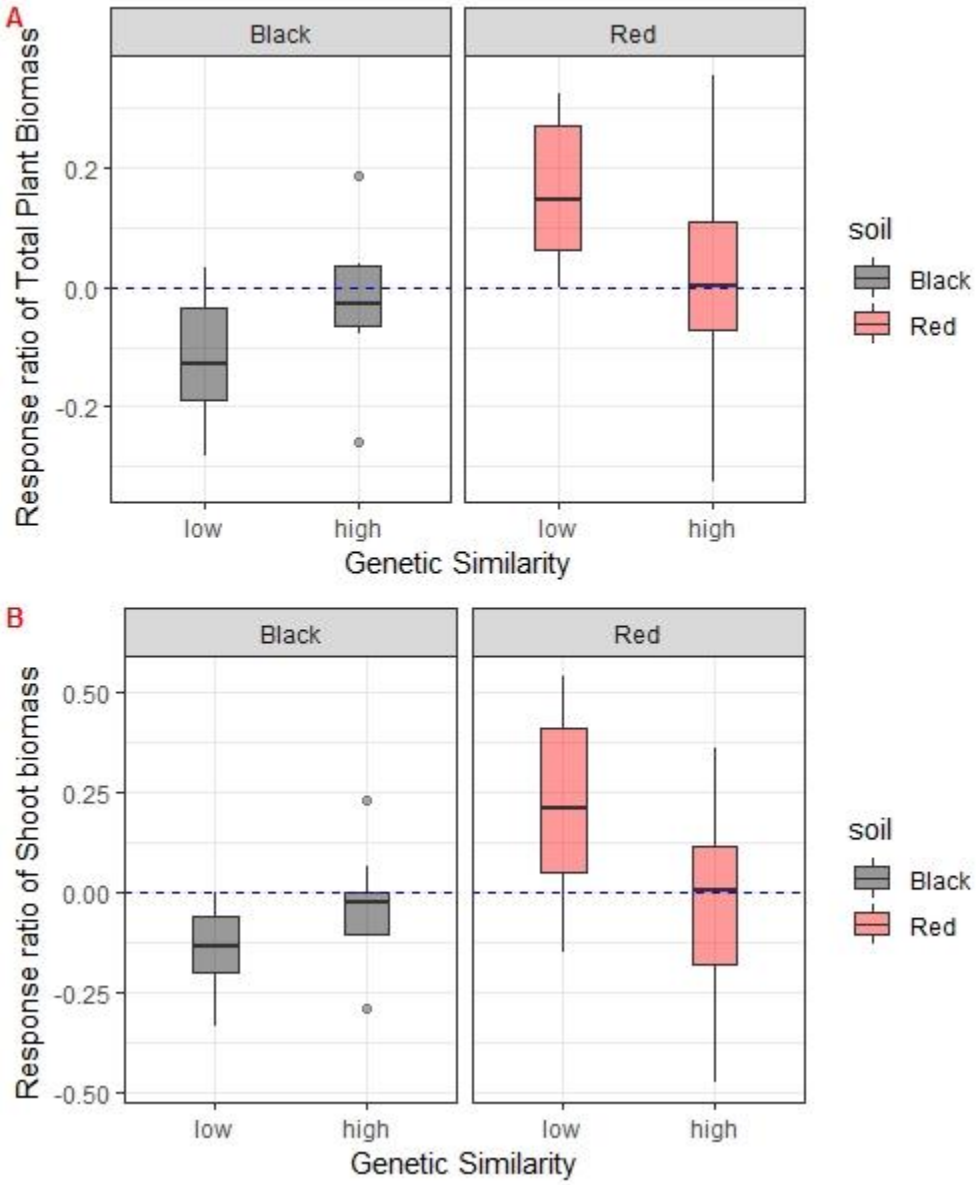

Figure 2. 7: Net co-inoculation effect on the (A) total plant biomass and (B) shoot biomass compared to single inoculations. The zero line depicts there is no net co-inoculation effect for response variable when the isolate is combined with another isolate. The positive response above the zero line indicates increased response of co-inoculation with pairs compared to on its own performance of strains and negative responses show that the performance of a strain on its own more effective than when co-inoculated with another strain. The effect of soil on response ratios of total biomass is significant at P=0.02 (Two-way ANOVA). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles. The vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range.

Regardless of the rhizobial similarity, plants grown in red calcarosol soil had an increase in co-inoculation response of their total plant biomass (~14%) compared to the plants grown in black vertosol soil (P=0.02, Two-sample t-test, Table 2.4).

Table 2. 3: Summary of two-way ANOVA showing the effects of rhizobial genetic similarity and type of soil the plants were grown in on the response ratios of nodule number, fixed nitrogen, Total nitrogen content and shoot, root and total biomass of the plant

Effect	DF	Response ratio of nodules		Response ratio of FixedN		Response ratio of TotN		Response ratio of Shoot biomass		Response ratio of Root biomass		Response ratio of Total biomass	
		F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Genetic Similarity	1	3.74	0.06†	1.36	0.25	0.37	0.54	0.62	0.43	0.2	0.65	0.16	0.69
Soil	1	0.25	0.62	0.81	0.37	4.96	0.04*	4.09	0.05†	1.28	3.68	6.26	0.02*
Genetic similarity*Soil	1	0.22	0.64	1.33	0.26	3.67	0.06†	4.69	0.04*	0.29	0.59	4.27	0.05†

Note: '*' indicates $P<0.05$ and '†' Indicates $P<0.1$

Table 2. 4: Summary of two sample t-test comparing the response ratios (RR) of plant measurements in red calcarosol and black vertosol soils

Effect	**DF**	***t*-value**	***P*- value**
RR of nodule number	26	-0.48	0.63
RR of fixed nitrogen	26	-0.89	0.37
RR of total plant nitrogen	26	-2.14	0.04*
RR of plant shoot biomass	26	-1.9	0.07†
RR of plant root biomass	26	-1.97	0.06†
RR of total plant biomass	26	-2.39	0.02*

Note: '*' indicates $P<0.05$ and '†' Indicates $P<0.1$

Rhizobial co-inoculation effects on nodule number and fixed amounts of N in plants did not significantly differ between two soil types ($P>0.05$, Two-sample t-test, Table 2.4).

2.3.3 Evaluation of the rhizobial genetic similarity on relative nodule occupancy of rhizobial and non-rhizobial endophytic (NRE) strains

I found that the genetic similarity of rhizobia had significant effects on the occurrence of non-rhizobial endophytes (NRE) ($P<0.01$, Analysis of Deviance Table (Type II tests) 2.6) where the high similarity rhizobial co-inoculations facilitated more NRE colonization (Table 2.5). There were differences in relative nodule occupancies of each of the rhizobial strains in a particular combination (Table 2.5). For example, the presence of *R. leguminosarum* RRI1220 was more frequent in nodules than the other rhizobial strains in a combination (~50%-80% of occurrence across both red and black soils). The strains RRI294 and RRI970 were almost absent across all co-inoculated nodules (Table 2.5). The number of rhizobial sequences observed in the low genetic similarity group were significantly higher than the number of rhizobial sequences observed in high similarity combinations ($P= 0.02$, Two-way ANOVA, Table 2.6).

Table 2. 5: Number of rhizobial and non-rhizobial sequences obtained (per ten clones) in nodules collected from different rhizobial co-inoculation treatments under low and high genetic similarity groups (Most frequent means more numbers detected and least frequent means less numbers detected).

Genetic Similarity	Soil type	Recovery of strains in nodules from a co-inoculation		No of most frequent *R. leguminosarum* strain	No of least frequent *R. leguminosarum* strain	Total no of rhizobial sequences	No of non-rhizobial sequences
		Most Frequently recovered strain	Least Frequently recovered strain				
Low	Black	RRI1220	RRI294	7	0	7	3
		RRI1220	RRI429	4	3	7	3
		RRI1220	RRI970	4	2	6	4
	Red	RRI1220	RRI294	8	1	9	1
		RRI1220	RRI429	5	0	5	5
		RRI1220	RRI970	4	1	5	5
High	Black	RRI429	RRI294	1	0	1	7
		RRI429	RRI970	1	0	1	9
	Red	RRI294	RRI429	3	3	6	4
		RRI548	RRI970	6	0	6	4
		RRI429	RRI970	2	0	2	8

Table 2. 6: Analysis of Deviance Table (Type II tests) showing the effect of rhizobial genetic similarity and the soil type on obtaining rhizobial or non-rhizobial genotype

Effect	**LR Chisq**	**DF**	***Pr* (> Chisq)**
Similarity	13.3323	1	<0.001 ****
Soil	0.0345	1	0.85
Similarity*soil	2.8501	1	0.09 †

Note: '**' for $P<0.01$, and '†' for $P<0.1$

Moreover, among the non rhizobial rDNA sequences obtained from nodules in each inoculation treatment, 10% to 90% of sequences were classified to bacterial species such as *Pseudomonas* sp., *Paenibacillus* sp., *Acidovorax* sp. and *Chryseobacterium* sp.

2.4 Discussion

This study compared the co-infection of multiple strains of *R. leguminosarum* to single strain infection of the field pea in terms of several performance indicators.

2.4.1 Genetically less similar rhizobial combinations are more efficient in root nodulation but not in total nitrogen assimilation in host plants

In line with my proposed hypothesis, plants with genetically less similar (between 70% and 90% similarity) rhizobial pairs had increased nodulation compared to those with highly similar (99% similarity) combinations in highly contrasting calcarosol and vertosol soils. Being genetically less related to each other may have enhanced the synergistic interactions by reducing inter-strain competition (Platt and Bever, 2009). Complex reciprocal signaling and recognition mechanisms have been reported (Barrett et al., 2015), however more work is required to identify the mechanisms leading to this synergy in this study. Despite having more nodules, field pea plants infected with genetically less similar rhizobial pairs did not have increased N fixation, total N or plant biomass. Similar to our findings, Rodríguez Blanco et al. (2010) observed that there was a high percentage of *Trifolium repens* and *Trifolium pratense* plant populations with more nodules but less N fixation

due to ineffective rhizobial communities. In my work, the genetically less similar rhizobial pairs are less efficient though they could produce more nodules in host plants. This indicates the importance of comparing individual strain efficiency in each of the measured plant responses. It is also possible that other plant related factors such as reduced carbon and oxygen flow to nodules may have led to poor N fixation efficiency of rhizobial endophytes (Kiers et al., 2006). We need further work to examine these mechanisms.

2.4.2 Comparison of the performance of rhizobial pairs with individual strains (Co-inoculation effect) revealed fewer nodules in closely related rhizobial pairs

When the genetic similarity of a rhizobial pair increased the co-inoculation effect (response ratio) plant nodule number decreased, suggesting that the higher the genetic similarity of *Rhizobium leguminosarum* strains in a combination, the weaker the nodulation, thereby supporting our hypothesis. The expected net N benefit from co-inoculation with highly similar rhizobial pairs is even less (~12%) than the net N benefits from single strain infection in legume hosts. This observation suggests that there could be an intense competition between closely related strains for similar nutrient resources when inoculated together compared to single inoculation (Burns and Strauss, 2011). This competition might have resulted in weak nodulation and N fixation performance in host plants as the rhizobial strains expends more energy on inter-strain competition. Further work is required to explore the signalling and metabolic pathways of these strain combinations to prove this hypothesis with field pea–rhizobium symbiotic system.

The use of two highly contrasting yet typical cropping soils highlighted the variation in plant N nutrition and biomass responses and the requirement to avoid generalizations regarding rhizobial performance in legume symbioses. Irrespective of rhizobial genetic similarity, the response ratio of co-inoculation significantly increased for total N content of the host plants in alkaline red soil compared to the individual strain performance. Theoretically it is suggested that multi-strain inoculations compensate for the environmental constraints (such as soil acidity/alkalinity or drought) which are hard to overcome by an individual strain alone (Somasegaran and Bohlool, 1990). Surrounded by multiple rhizobial strains (functionally diverse) with varying symbiotic N fixation efficiencies is giving added advantage for legume hosts in stressed soils (Simms and Taylor, 2002).

Environmental stresses including soil acidity and alkalinity, low nutrient levels, and low water availability would negatively affect both rhizobia and legume partners leading to less N fixation, poor crop yields and loss of plant vigor (Zahran, 1999). There were increased plant shoot and total plant biomass response ratios of genetically less similar rhizobial co-inoculants in red calcarosol soil. Most of the red calcarosol soils in Mallee region in Southern Australia are highly alkaline (pH 7.5-8.5) (Nuttall et al., 2003), depending on the depth of the soil profile, and often result in nutrient (such as Zinc) deficiencies. (CSIRO, 2016, Hafeez et al., 2013). Further work is needed to determine whether an increased plant biomass response to the rhizobial co-inoculation in calcarosol is due to a trait of rhizobia in low similarity combinations, such as synergistic interactions, or if there can be any antagonistic effects when the high genetic similarity strains co-infect the legume host (Heath and Tiffin, 2007). I suggest that more data on persistence of these different rhizobial similarity groups in different stressful conditions and success of their N fixation under those circumstances would be necessary to assess co-inoculation success. As described by McCallum et al. (1999), field pea is grown in a large area of land with black vertosols in Wimmera of VIC, Australia, expecting more N fixation. Although the pea plants grown in black vertosol soils in this experiment had more total N and total biomass, my data was not sufficient to show whether this soil type aids in increasing rhizobial nodulation and N fixation.

2.4.3 Variation in the occurrence of non-rhizobial endophytes (NRE) in different rhizobial pair-wise inoculations

There were significant numbers of non-rhizobial nodule endophytic (NRE) sequences that were identified as *Pseudomonas* sp., *Paenibacillus* sp., *Acidovorax* sp. and *Chryseobacterium* sp. that reside together with rhizobial strains in host nodules. Moreover, the genetic similarity of rhizobial pairs changed the percentage occurrence of these endophytes. The high similarity rhizobial pair-wise inoculations significantly facilitated NRE colonization regardless of soil type. The intense competition between high similarity rhizobial isolates may result in weaker colonization of the host root providing greater access of opportunistic NRE to available nodule spaces during the nodulation process. One of the most frequently detected NRE in high similarity rhizobial inoculations was *Pseudomonas* sp.

In a previous study, Egamberdieva et al. (2010) co-inoculated *Rhizobium galega bv. orientalis* with cellulose producing *Pseudomonas* which increased nodulation and N in *Galega orientalis* Lam.

(fodder galega) whereas the cellulose negative *Pseudomonas* had no significant effect on nodulation or N uptake. Further, it was suggested that these cellulases could act as supplementary chemicals to help rhizobia to gain host entry through root nodules. Therefore, the NRE communities observed in my work could also interact directly and/or indirectly in legume symbiosis or have no effect at all and just are able to occupy nodules because there is available nodule space which could be tested in a future study.

In this study, the rhizobium strain most frequently recovered in the nodule was RRI1220 and the least was RRI294. The variation in the ability of root colonization could be due their differences in symbiotic traits where RRI1220 could have more resistivity in biotic (other microbial) and abiotic (soil) stresses. However, this study had the potential limitation of DNA from a low number of individual nodules to identify the occurrence of RRI strains, which could be addressed in future studies by increasing the number of nodules for bacterial DNA extractions.

2.4.4 Overall understanding of this work, limitations and directions for future work

The work conducted in this experiment shows that the identification and management of resident rhizobial communities is important to promote legume crop productivity because the effective rhizobial partners are limited in cropping soils (Naamala et al., 2016, Friesen, 2012). My work on inter-strain interactions of *R. leguminosarum* adds more value when selecting multiple rhizobial strains for legume inoculation. For example, the current findings showed that coupling genetically similar strains did not necessarily enhance plant biomass gain and nitrogen assimilation in field pea plants. There was also a significant effect of soil type on rhizobial performance. It is also suggested that the interactions between the rhizobial strains and the plant hosts are potentially complex which can be influenced by several environmental variables. Further, an accurate understanding of rhizobial interactions is required to evaluate the efficiency of multi-strain inoculants since individual strains might possess variation of symbiotic traits such as improved colonization efficiency which we observed with RRI1220 in my study. Therefore, I suggest more future work to investigate the individual nodule level interactions of *R. leguminosarum* strains in field pea hosts to understand the inter-strain competition in an additional level of N symbiosis.

This study further suggests that soil properties (such as pH, available nutrient content) could be good indicators of effective rhizobial interactions. For example, the field pea plants grown in

nutrient-limited red calcarosol soil gained more biomass and accumulated more nitrogen when inoculated with genetically dissimilar rhizobial strains. Future work could attempt to investigate the effects of these abiotic constraints on rhizobial interactions to gain more nitrogen benefits. In conclusion, my work revealed that the co-inoculation with genetically less related R. *leguminosarum* strains could increase the nodulation and plant shoot biomass of field pea hosts compared to the performance of strains on their own. More broadly, research is also needed to determine whether all strain combinations enhance nodulation and N fixation depending on how similar they are to each other. In order to achieve sustainable legume nitrogen symbiosis, this study demonstrates that inter-strain interactions of rhizobia are soil type specific and may or may not lead to enhanced performance of legumes.

Chapter 3: Low frequency of infections by multiple *Rhizobium leguminosarum* strains in a single nodule during rhizobial N symbiosis in field pea

3.1 Introduction

Rhizobial nitrogen (N) fixation enables the legume hosts to thrive in N-limited soils in agricultural lands all over the world. According to Herridge et al. (2008), the global N fixation in agricultural systems accounts for 50-70 Tg N yr^{-1} which indicates a great alternative to replacement of inorganic N fertilizer. In nature, legume plants can be colonized by multiple strains of rhizobia (Oono et al., 2009) simultaneously exposing a plant to symbionts that may differ in their capacity to fix N (West et al., 2002, Carelli et al., 2000). Although in most cases, mutualisms are beneficial to both the partners by facilitating each other's growth and survival (Bronstein, 2009), multiple rhizobial partners trying to nodulate the same legume plant could create intense inter-strain competitive pressure to acquire limited host resources (Bourion et al., 2017). This could lead to the colonisation of highly competitive but ineffective nitrogen fixers in host plants which creates negative impact on plant N symbiosis (Barrett et al., 2015).

In general, rhizobial symbionts of the same species may include both effective and ineffective rhizobial strains, the latter fixing less or no N in the host plant despite successfully nodulating the host (Heath and Tiffin, 2009). Previous studies have explored the plant's ability to penalize the ineffective rhizobia by sanctioning the ineffective nodules (Kiers et al., 2003) or by selecting only the effective ones at the point of entry (Heath and Tiffin, 2009). Most of these studies have compared the effective rhizobial strains with their laboratory mutants lacking N fixing genes (such as *nif* genes). Some resident rhizobial populations in cropping fields are well-adapted to extreme environmental conditions such as drought and low pH (Brockwell et al., 1995). Failure of efficient N fixing commercial inoculants when introducing to new cropping environments could be that their low adaptation to particular soil/ environmental conditions and competitive dominance of resident rhizobial populations adapted to such conditions (Rodríguez Blanco et al., 2010 and Triplett and Sadowsky 1992). These studies show that the commercially introduced rhizobial strains need to be efficient in nodulation to win the intense competition of the ineffective strains in the rhizosphere. Competition existing among rhizobial strains can be used to predict outcomes of inter-strain interactions in rhizobial-legume symbiosis (Kiers et al., 2007).

Genetic diversity of resident soil rhizobia can be related to nitrogen fixation efficacy in the legume hosts (Mutch and Young, 2004). Since the ineffective rhizobia can be widespread (Simms and Taylor, 2002), the effective rhizobia need to be sufficiently competitive to override the ineffective strains to nodulate the host legumes.

Although there is a possibility that the host plant can sanction the non-functional and poorly-functional nodules , in a mixed rhizobial infection (Kiers et al., 2006) , ineffective N fixers can avoid host plant sanctions by aligning with more effective rhizobia in the same nodule (Denison, 2000). Further, these ineffective rhizobia were defined as 'free riders' where they gain resources of the host plant without giving fixed nitrogen. However, when the two rhizobial strains in a mixed infection belong to the same species we could expect more competition for nodule resources where one strain could possibly outcompete the less competitive strain. As described by Checcucci et al. (2016), one way of inter-strain competition occurs when two or more rhizobial strains reside in one single nodule. Mixed nodule infection was observed in different legumes such as in soybean (12%-32%) (Moawad and Schmidt, 1987), lupin (~1%) (Simms et al., 2006), alfalfa (2.9%) by Van Berkum et al. (2012) and 27%-100% by Checcucci et al. (2016) in laboratory conditions.

The current study evaluated the extent to which mixed infection by multiple *Rhizobium leguminosarum* strains occurs in *Pisum sativum* (field pea) symbiosis, both within and between nodules of single plants. Field pea was selected as the host legume plant because it is a major legume crop in Australia where the annual production is about 0.3 million tonnes (Poblaciones and Rengel, 2016). Rhizobial N fixation is limited in most field pea crops (Jensen, 1987) due to ineffective resident rhizobial populations. I predicted that ineffective rhizobial populations and the competition existing among ineffective strains and effective inoculant strains for nodulation could be a major limiting factor for successful N symbiosis. Howieson and Ballard (2004) claimed that intra-specific competition of rhizobia for nodulation is higher than inter-specific competition. Strains of the same species might be having similar traits of plant signal reception for nodulation thus similar strains could compete to access the receptor molecules produced by the plant. It was predicted that closely related rhizobial strains, either within individual nodules or between nodules on the same plant, would ultimately lead to poor nitrogen fixation rate and reduced yield in legume crops (Triplett and Sadowsky, 1992, Friesen, 2012). To determine whether the genetic similarity

of rhizobial combinations significantly affected legume N fixation, I used several *Rhizobium leguminosarum* strains in pairs of varying genetic similarity observed using 16S rDNA sequences.

Previous work (including my work in chapter 2) showed that inoculation with two rhizobial strains of high genetic similarity can lead to reduced nodulation of field pea hosts in comparison to inoculation with each individual strain. In this chapter, I assessed the extent that dual infection occurs within a root system and within individual nodules. I hypothesized that rhizobial pairs with high genetic similarity would exhibit higher inter-strain competition and would be less likely to co-exist inside a nodule and among multiple nodules on the same root system (Figure 3.1). One hypothesized scenario for this competition could be that high levels of genetic relatedness between strains indicate having similar traits for carbon resource acquisition and establishment in nodule space (Hibbing et al., 2010). Hence, I expected a low level of mixed nodule infections of these highly similar pairs compared to low similarity pairs. Furthermore, I expected that the competition between high similarity rhizobial pairs would negatively affect the nodulation, N fixation and plant biomass due to investment in their own competition instead of nitrogen fixation.

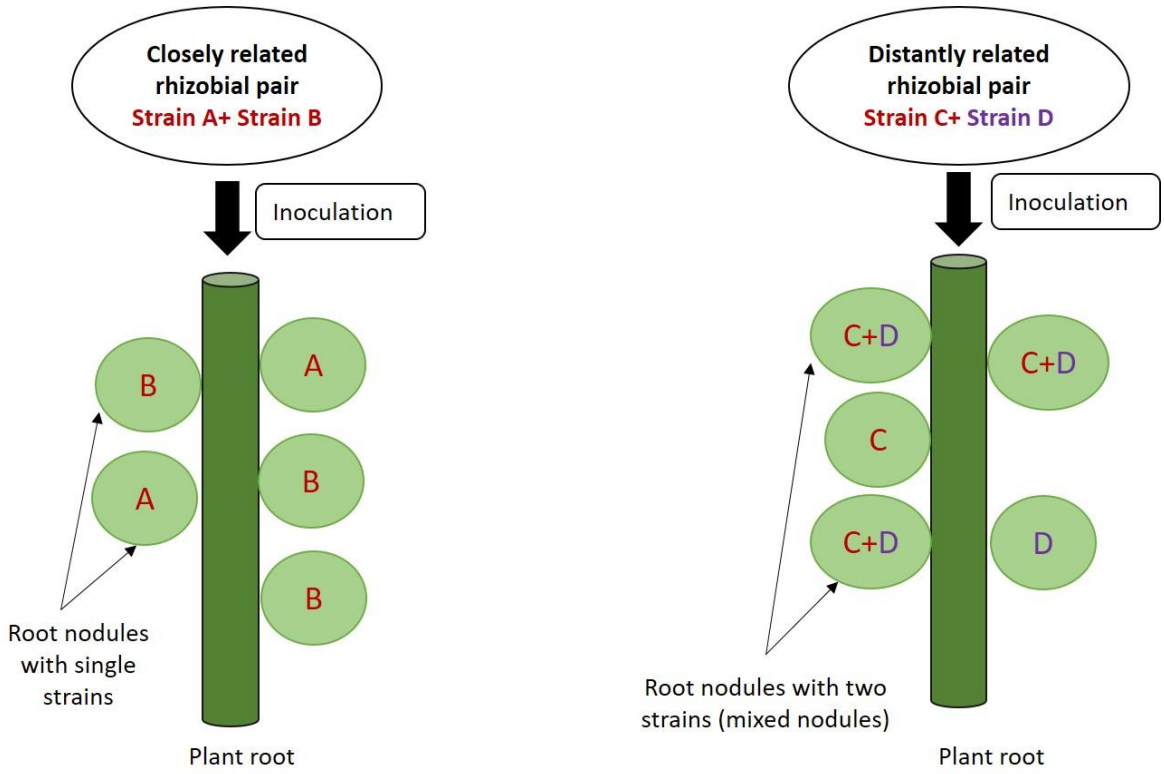

Figure 3. 1: Hypothetical diagram of predicted scenarios for inoculation with closely and distantly related rhizobial pairs in pea root systems.

3.2 Materials and methods

3.2.1 Rhizobial strain selection using ERIC PCR for pair-wise inoculation

Eleven *Rhizobium leguminosarum* strains were obtained from the Department of Primary Industries Victoria culture collection at Rutherglen, VIC, Australia. ERIC fingerprinting (Leung et al., 2004) was used to generate banding patterns of these strains. The rhizobial DNA was amplified using ERIC sequence primers; ERIC 1R – 5'-ATG TAA GCT CCT GGG GAT TCA C-3' and ERIC 2 – 5'-AAG TAA GTG ACT GGG GTG AGC G-3' (Versalovic et al., 1991) (BIONEER Corporation, Korea). Each PCR reaction contained 20 µl of master mix: 4 µl of 5x MyTaq™ Reaction Buffer (Bioline Pty Ltd., Australia) which includes 5 mM dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers, 0.2 µl of MyTaq™ DNA Polymerase (5U/µl) (Bioline Pty Ltd, Australia), 0.4 µl of ERIC 1R (25 pmol), 0.4 µl of ERIC 2 (25 pmol), 13 µl of PCR grade water and 2 µl of template DNA. PCR cycling conditions were as follows: initial denaturation at 95°C for 3 minutes, then 35 cycles of denaturation at 95°C for 20 seconds, annealing at 50°C for 15 seconds, primer extension at 72 °C for 90 seconds and final extension at 72°C for 5 minutes. The resulting PCR products were run in 1.5% Agarose gel (Vivantis Technologies, Malaysia) stained with Invitrogen™ SYBR™ Safe™ DNA Gel Stain (S33102) (Thermo Fisher Scientific, Australia) at 85 V for 90 minutes. The gel was observed in Gel Doc™ (Bio-RAD, Australia) using Image Lab™ Software 3.0. (see Results Section- Figure 3.3.2). Among 11 rhizobial isolates, five showed unique banding patterns that could be used to differentiate among them and be used in the experiment.

3.2.2 Constructing phylogenetic tree for assessing genetic similarity of selected *R. leguminosarum* strains in the study

Single colonies from each of the rhizobial strain were picked up with sterile needles and lysed using thermocycler (Dyad Peltier Thermal Cycler) at 94°C for 10 minutes. 16S colony PCR was performed for these lysed colonies using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers (Suzuki and Giovannoni, 1996). For more details on PCR reaction see chapter 2 Materials and methods – 2.2.1. The amplified DNA was purified using AGENCOURT®AMPURE® PCR purification kit. The purified DNA samples were sequenced using 518F (5' CCAGCAGCCGCGGTAATACG 3') and 800R (5' TACCAGGGTATCTAATCC 3') sequence primers (Lamsal et al., 2012) at Sanger sequencing

facility at HIE (3500 Genetic Analyzer 8ch RUO-6220010, Hitachi High Technologies Corporation, Tokyo, Japan). The 16S rDNA sequences (obtained from SANGER sequencing) of 11 *Rhizobium leguminosarum* strains were used to build the phylogenetic tree. The sequences were aligned using the program Multiple Sequence Alignment (MAFFT) version 7.0 (Katoh and Standley, 2013) and the resulting alignments were used with 1000 bootstrap replications to obtain the maximum likelihood tree in phylogenetic tree constructing software MEGA 7.0.

3.2.2 Seed germination and plant growth in plate-based substrate system

The seeds of the *Pisum sativum* (field pea) cultivar Wharton (Hart Bros Seeds Pty Ltd, Junee Reefs NSW, Australia) was used in this experiment. Seeds were surface-sterilized as described in Chapter 2, section 2.2.2 – Materials and methods. Pea seeds were germinated on sterile moist paper towels for 5 days under dark conditions (Hameeda et al., 2008). Germinated seedlings were carefully transferred to a sterile sand: vermiculite substrate (3:1) prepared in 145 mm sterile Petri plates. The plates were drilled with a slit to allow the shoot to develop **(**Figure 3.2**)** and the rest of the plate was covered with aluminium foil to avoid direct light damage to the root system (in case roots appear through the shallow sand substrate).

Figure 3. 2: Development of sand: vermiculite media on 145 mm Petri plate with germinated field pea seedling (5 days old).

Each treatment (described below) contained six biological replicates. Plants were watered carefully with 20 ml of sterile milliQ water every two days to maintain plants in sand substrate and 70% fan

speed to maintain 60% humidity. Modified N-free Hoagland's solution (Hoagland and Arnon, 1950) (Appendix 3) was added once in a week to fulfil plant nutrient requirements.

3.2.3 Rhizobial inoculum preparation and inoculation of seedlings

Rhizobial inocula were prepared using the same protocol described in section 2.2.2- Materials and methods in Chapter 2 (Somasegaran and Hoben, 1985). After three days of incubation, liquid cultures were inoculated to 10 days old seedlings as single and pair-wise combinations (Table 3.1, Figure 3.3). Emergence of root nodules was checked a week after the inoculations (supplementary Figure S3-1).

Figure 3. 3: Inoculation of pea seedling grown in sand: vermiculite media using broth cultures of selected RRI strains under sterile conditions.

The plants were grown for 10 weeks under 21℃, ambient CO_2 (400 ppm) and 60% humidity at light intensity of 200 µmol.m^{-2}.s^{-1} for photoperiod of 13 hours in controlled growth environment (BioChambers- INBIO BIOINSIGHT Pty Ltd.) before harvesting (Yu et al., 2012).

3.2.4 Seedling harvesting, counting nodule number and measuring root and shoot biomass

Ten-week-old plants were harvested and washed carefully with sterile milliQ water to remove attached sand and vermiculite particles on roots. Nodule numbers were counted and recorded. There were no nodules present in any of the uninoculated control plants. Ten nodules were inserted into 12 ml glass vials and crimped with aluminium cap and a rubber septum for acetylene reduction

assay (ARA) (Refer to section **3.2.4.1** for more details on ARA). The remaining nodules were stored at -20℃ for further analysis. Plant shoots and roots were dried at 70℃ for 48 hours, after which shoot and root dry weights were recorded.

3.2.4.1 Acetylene Reduction Assay

Potential N fixation was assessed with the acetylene reductions assay (Hardy et al., 1968). Briefly, for each replicate, ten nodules were enclosed in a gas tight vial as in Figure 3.4 (Somasegaran and Hoben, 1985).

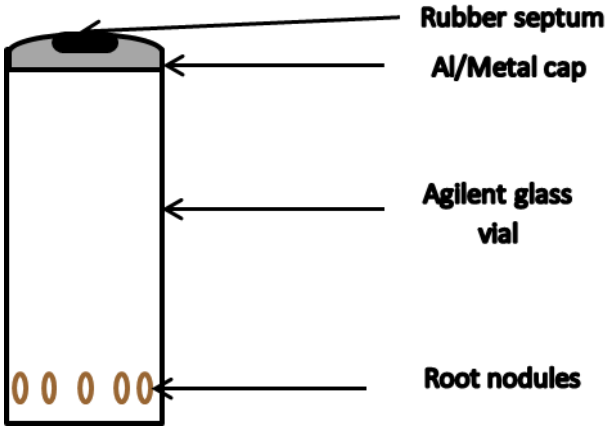

Figure 3. 4: Agilent glass vial used for acetylene reduction assay enclosed with freshly harvested root nodules. Volume of the vial is 2 ml and the dimensions are 12 mm x 32 mm (12 mm cap).

Approximately 1-5% (1 ml) of the air in the vial was replaced with acetylene and incubated for one hour at room temperature (due to the action of nitrogenase enzyme C_2H_2 converts to C_2H_4). After incubation period, 1 ml of gas was removed from the nodule containing vial automatically using the robotic hand of the gas analyser (GC) and fed to relevant port of a GC (GC system 7890A, Agilent Technologies Inc, USA) to analyse the ethylene produced per unit time. Ethylene analysis was performed using an Agilent 7890A gas chromatograph fitted with an HP-PLOT/Q column (30 m x 0.53 mm x 40 µm), a multimode inlet and a flame ionisation detector. Hydrogen was used as the carrier gas at a flow rate of 6 ml/min. The resulting ethylene peaks were quantified using a standard curve prepared from a 1000 ppm ethylene standard.

3.2.6 Molecular identification of *R. leguminosarum* strains in harvested nodules

Rhizobial DNA was extracted from root nodules using Isolate II Plant DNA kit BIO 52070 (Bioline Pty Ltd, Australia) following the manufacturer's instructions. Five nodules per plant were subjected to molecular identification (ERIC PCR). Nodules from single strain inoculated plants were also subjected to ERIC PCR to confirm no cross contamination (five nodules per plant). Extracted DNA concentrations were measured using NanoDrop 2000 Micro-volume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Delaware USA). ERIC PCR was performed using extracted DNA samples in BIO-RAD Dyad® Peltier thermal cycler (BIO-RAD, Australia) as previously described (section 3.2.1- Materials and methods in this chapter).

3.2.6 Data Analysis

Statistical analyses used R version 3.5.1 (R Development Core Team, 2016). Data from 72 plants (both single and multi-strain inoculations) were used and each treatment had six biological replicates. Data were analysed using (1) linear models fit to average responses of all the treatments and (2) linear models to fit into response ratios of rhizobial pairs compared to their individual strain performances (Yuan and Chen, 2015). The normality and homoscedasticity of residuals in linear models were assessed using diagnostic plots. The figures of plant responses (and response ratios) were constructed using the package 'ggplots' (Wickham, 2009) in R 3.5.1 demonstrating the responses of each rhizobial-pair classified into two major genetic similarity groups (low and high). To test the hypothesis of mixed nodule infection, the data generated from ERIC fingerprinting was evaluated. Since the mixed nodule infections were a rare case to detect, we looked whether the rhizobial genetic similarity has any effect on the mixed nodule colonisation using generalized linear modelling (GLM- binomial distribution in R).

The effect of genetic similarity on observed plant responses such as total nodule number per plant, nitrogenase activity (Ethylene µmol per hour per plant), total plant biomass, total and average nodule biomass data was evaluated using linear mixed effects modelling. The function 'lmer' in the package 'lme4' (R 3.5.1) was used to fit linear mixed effects models (using the 'strain combination' as a random effect). *P*-values were calculated using Anova (Type II Wald F tests with Kenward-Roger DF).

To determine the performance of a rhizobial strain in a combination compared to on its own, response ratio values of each measured plant response were calculated using the following equation:

Response ratio = ln (response [combination]/response [single strain])

The log response ratios were fit to linear mixed effects model ANOVA (Type II Wald F tests with Kenward-Roger degrees of freedom) to determine whether the genetic similarity of *R. leguminosarum* has significant effects on response ratio values. To test whether the 'high' and 'low' similarity groups were significantly different in each of the measured plant responses, two-sample t-tests were calculated. Moreover, a one-sample t-test was performed to test whether the measured plant responses were greater when the strains were inoculated as a pair compared to single-strain inoculation. To determine whether there was any significant variation between rhizobial combinations within each similarity group, I performed post hoc analyses of multiple comparisons using the R package 'emmeans' (method = Tukey).

3.3 Results

3.3.1 Determining the genetic similarity of R. *leguminisarum* strains in this study

Of the five *R. leguminosarum* strains, seven pairs were selected according to their genetic similarity (Figure 3.5 and Table 3.1) and based on the ability of identifying them separately in ERIC fingerprinting (Figure 3.6).

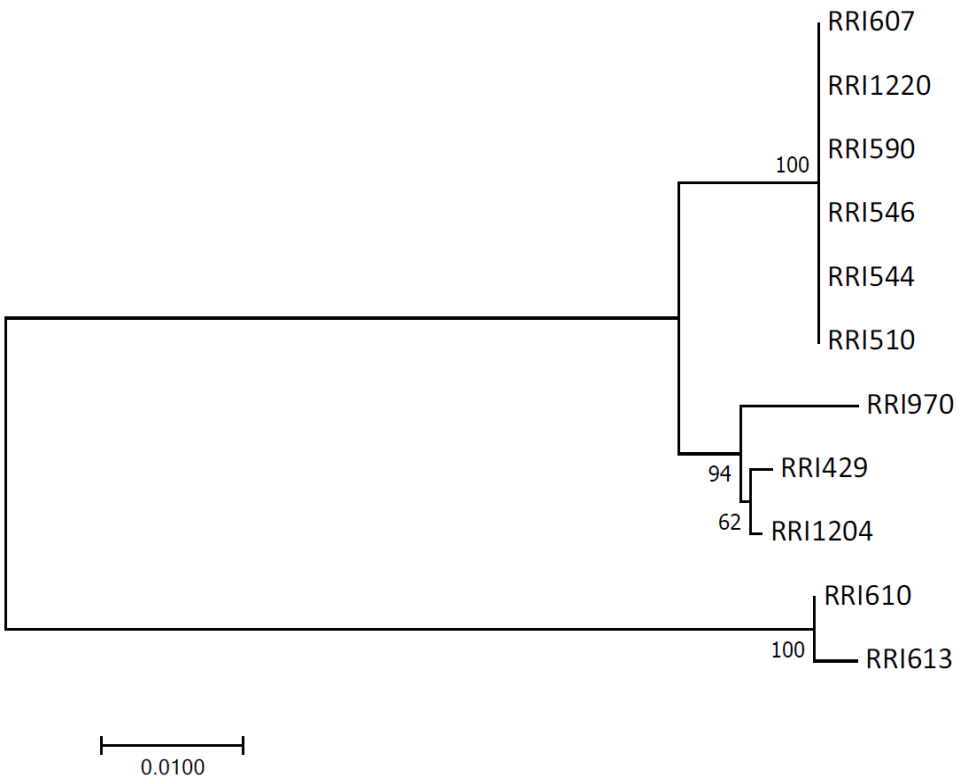

Figure 3. 5: Maximum likelihood tree for rRNA sequences of R. *leguminosarum* RRI strains using HKY (Hasegawa-Kishino-Yano) model with 1000 bootstrap replications. The tree was built in Molecular Evolutionary Genetics Analysis Version 7.0 (MEGA7). The branch length represents the evolutionary time between two nodes. The percentage numbers associated with each node indicates the bootstrap support to show how reliable the branching split is. The scale bar indicates the proportion of bases changing along the branches.

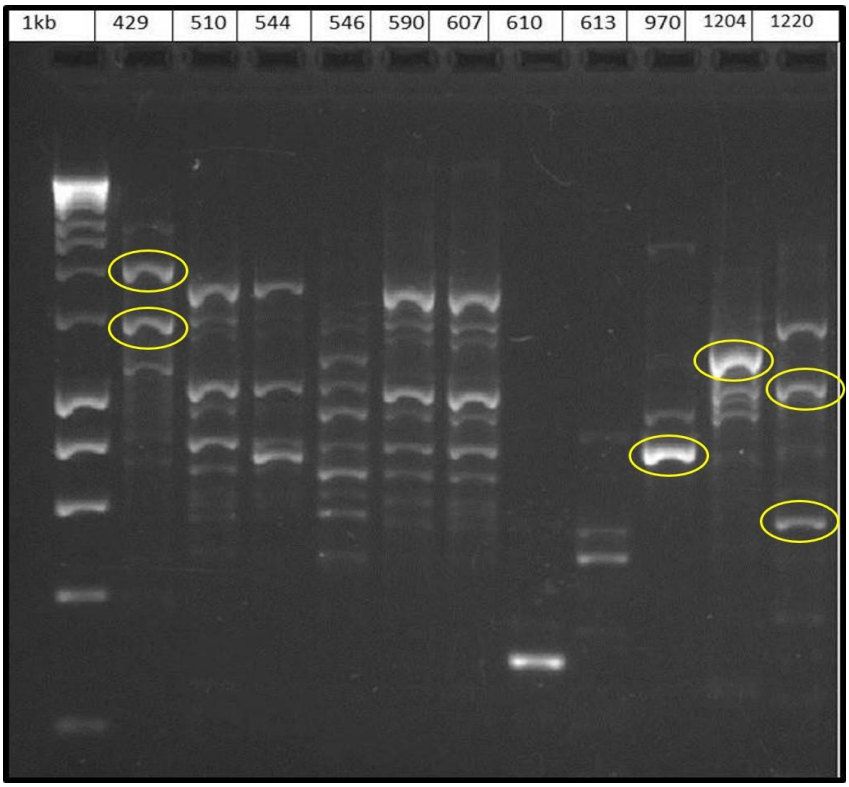

Figure 3. 6: Gel image showing ERIC fingerprints of *R. leguminosarum* strains with 1kb Ladder (Bioline Pty Ltd, Australia). The unique band sizes for identifying the strains of interest are circled in yellow: RRI429-1500bp and 2000bp, RRI970- 800bp, RRI1204- 1200bp and RRI1220- 1000bp and 500bp.

Table 3. 1: RRI Strain combinations based on their genetic similarities extracted from *R. leguminosarum* phylogeny observed in Figure 3.5.

Low similarity	**High similarity**
RRI 1220/ RRI 970	RRI 1204/RRI 970
RRI 1220/RRI 1204	RRI 429/RRI 1204
RRI 1220/RRI 429	RRI 429/RRI 970
RRI 429/ RRI 546	

3.3.2. Limited evidence for mixed nodule infection in the *Rhizobium leguminosarum*- Field pea symbiosis

Occurrence of mixed nodules was rare and only found in two plants treated with RRI429/RRI546 and RRI1220/RRI1204 (Table 3.2). This was not in line with my expectation that less genetically

related *R. leguminosarum* strains would more frequently form mixed nodule infections. Using ERIC fingerprinting, I observed occurrence of mixed infections in only two plants.

Table 3. 2: Average nodule occupancies of *R. leguminosarum* isolates in pair-wise inoculation treatments

Genetic similarity	Treatment	Most Frequently observed isolate	Least Frequently observed isolate	Average no of nodules with most frequent isolate	Average no of nodules with least frequent isolate	No of Mixed nodules per plant with both of isolates
low	429-1220	1220	429	23	4	0
low	1220-1204	1220	1204	45	0	1
low	1220-970	1220	970	41	2	0
low	429-546	546	429	44	2	1
high	1204-970	1204	970	13	8	0
high	429-1204	429	1204	18	1	0
high	429-970	429	970	22	1	0

Multiple rhizobial strains were detected in nodules from the same root system for 25% and 14% of plants inoculated with pairs of strains with low and high genetic similarity, respectively (e.g. supplementary Figure S3-2, Appendix 2). These percentages were not significantly different from each other ($P_{\text{genetic similarity}}$=0.37, two sample t-test).

3.3.3 Effects of rhizobial genetic similarity and particular rhizobial combinations on field pea plant response variables

The effects of co-inoculation were described as response ratios comparing single and multi-strain treatment plant responses. Further, I have examined the significant effects of particular strain combination(s) on measured response variables to account for the variation observed in each similarity group.

3.3.3.1 Pea plants inoculated with genetically less similar rhizobial pairs had more root nodules compared to ones with highly similar strains

Plants with genetically less similar pairs had 50% more nodules compared to the plants with highly similar rhizobial pairs (P=0.01, one-way ANOVA (Type II Wald F tests with Kenward-Roger DF),

Table 3.3). All three R. *leguminosarum* combinations used in the high genetic similarity group did not show any significant variation in nodule number with each other (*P*> 0.05, Multiple comparisons, Figure 3.7). In the low genetic similarity group, the pair RRI429/546 had 50% higher nodules compared to the pair RRI429/1220 (*P*=0.04, Multiple comparisons) whilst all other combinations in the low similarity group did not significantly differ in their performance among each other (*P*>0.05, multiple comparisons, Figure 3.7).

Table 3. 3: Summary of one-way ANOVA (Type II Wald F tests with Kenward-Roger DF) showing the effects of genetic similarity of *R. leguminosarum* strain combinations on plant responses

Response	**F**	**DF**	**Df.res**	***Pr*(>F)**
Nodule number/plant	12.43	1	4.96	0.01*
Ethylene µmol/hr/plant	2.98	1	4.94	0.02*
Total nodule biomass	10.49	1	4.73	0.03*
Average nodule mass	0.25	1	5.00	0.64
Total plant biomass	5.6	1	4.83	0.06†

Signif. codes: '*****' *P*<0.05, '†' *P*<0.1

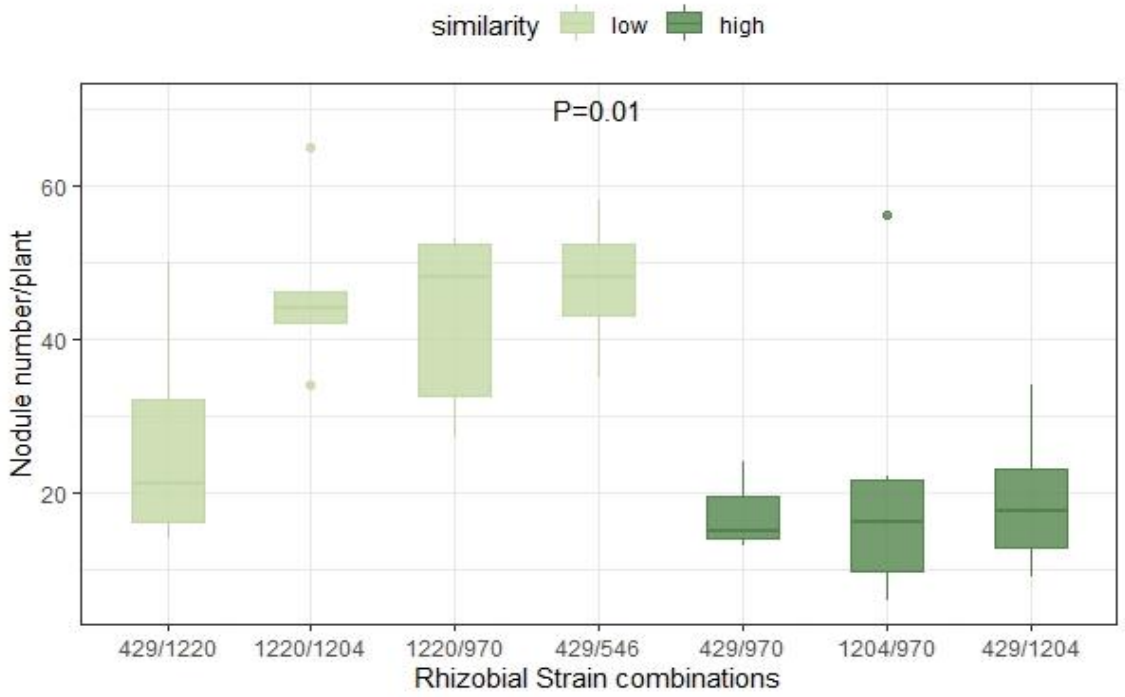

Figure 3. 7: Variation in the number of nodules produced in pea plants co-inoculated with pairs of *Rhizobium leguminosarum* strains differing in their degree of genetic similarity (based on 16S rDNA sequences). Results shown are for n=6 (biological replicates) and ordered from lowest to highest average response in each similarity group. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range. $P_{\text{genetic similarity}}$ was 0.01 (one-way ANOVA (Type II Wald F tests with Kenward-Roger DF)).

All the strain combinations (across both genetic similarity groups) had 50%-100% increase in nodulation response compared to the performance on their own Response ratio of nodule number >0, Figure 3.9).

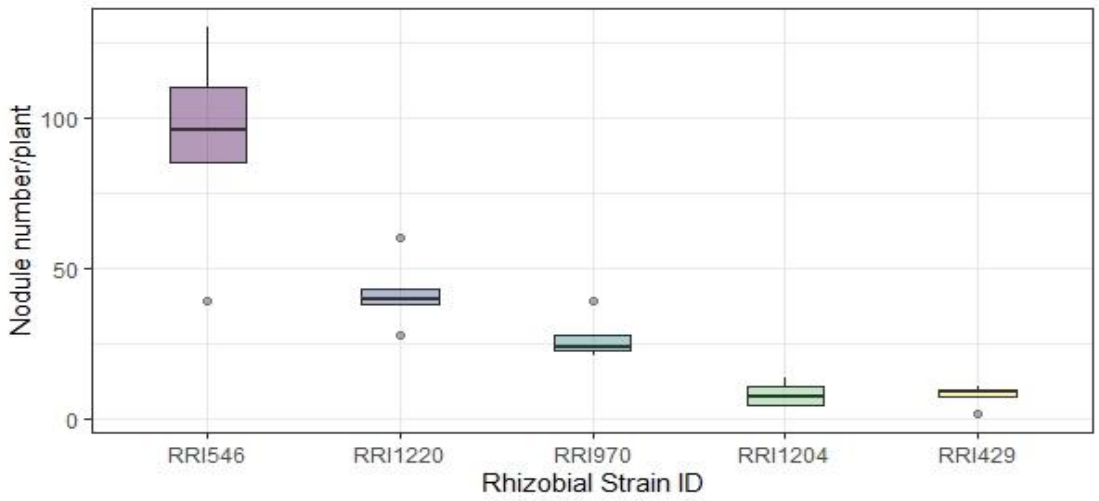

Figure 3. 8: Variation in the number of nodules produced in pea plants inoculated with single strains of *Rhizobium leguminosarum*. Results shown are for n=6 (biological replicates) and ordered from lowest to highest nodule number response in each similarity group. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range.

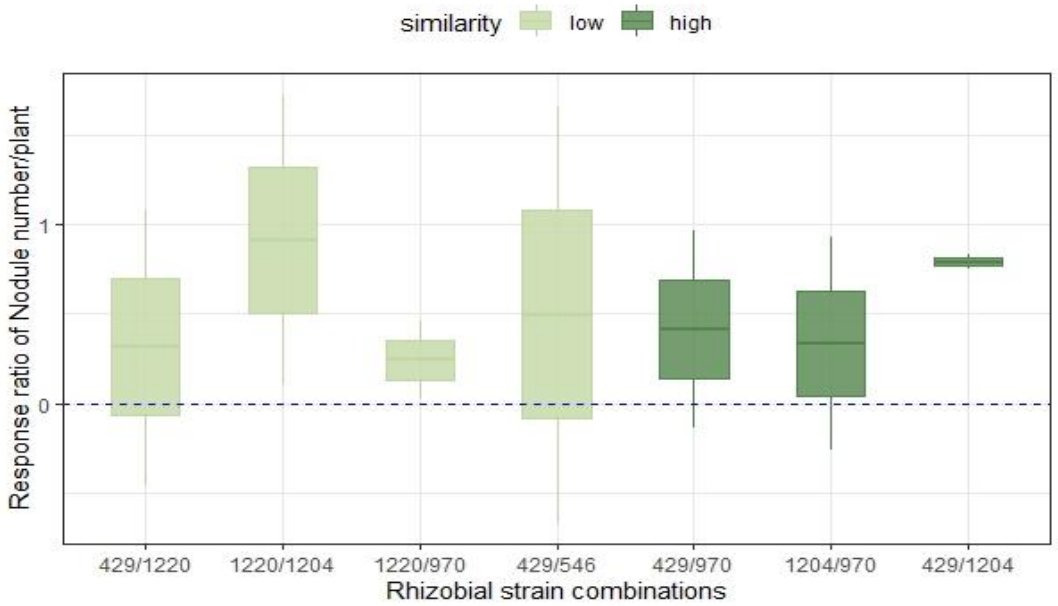

Figure 3. 9: Net co-inoculation effect (measured as response ratios) on the total nodule number per plant. The zero line depicts no net co-inoculation effect for response variable when the isolate is combined with another isolate. There were no significant differences between two similarity groups (P>0.05, calculated with one-way ANOVA (Type II Wald F tests with Kenward-Roger DF)) as well as among the treatments of the same similarity group (post-hoc analyses).

The net co-inoculation response of plant nodule number (Figure 3.9) did not differ between the two rhizobial genetic similarity groups (*P*>0.05, one-way ANOVA (Type II Wald F tests with

Kenward-Roger DF), Table 3.4). In the high genetic similarity group, RRI429/1204 had 2-fold higher nodulation response ratio compared to nodule numbers on their own (Figures 3.8 and 3.9). In contrast, the combinations RRI429/1220 and RRI429/546 (low similarity pairs) show 3-fold increase compared to RRI429 on its own and 50% decrease compared to RRI1220 and RRI546 on their own respectively (see Figures 3.8 and 3.9).

3.3.3.2 Nitrogen fixation rate was higher in field pea plant inoculated with genetically less similar rhizobial combinations

In general, rhizobial combinations with low genetic similarity had relatively more N fixation (~55% greater) compared to the pairs with high similarity (P=0.02, one-way ANOVA (Type II Wald F tests with Kenward-Roger DF), Table 3.3, Figure 3.10). There were no significant differences in the rate of nitrogen fixation (as nitrogenase activity) between the strain combinations in the low similarity group (P=0.38, Multiple comparisons, Figure 3.10). Moreover, RRI1204/970 had the lowest N-fixation rate in pair-wise inoculations (close to no fixation).

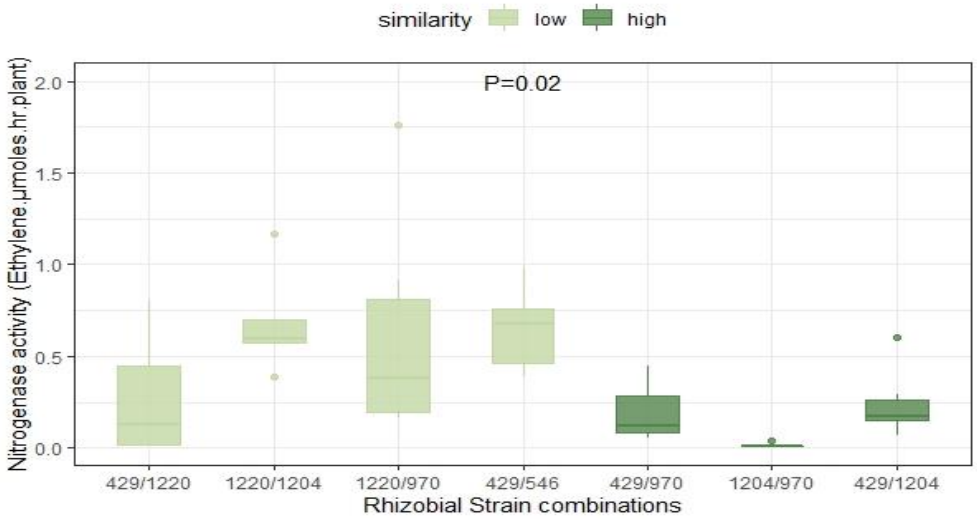

Figure 3. 10: The rate of rhizobial nitrogen fixation in root nodules of pea plants co-inoculated with pairs of *Rhizobium leguminosarum* strains differing in their degree of genetic similarity (based on 16S rDNA sequences). Results shown are for n=6 (biological replicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range. $P_{\text{genetic similarity}}$ was 0.02 (one-way ANOVA (Type II Wald F tests with Kenward-Roger DF)).

Table 3. 4: Summary of one-way ANOVA (Type II Wald F tests with Kenward-Roger DF) showing the effects of genetic similarity of *R. leguminosarum* strain combinations on response ratios of strain combinations (co-inoculation effect)

Response	F	DF	Df.res	Pr(>F)
Response ratio of total nodule number/plant	0.002	1	5	0.96
Response ratio of ethylene µmol/hr/plant	0.02	1	5	0.89
Response ratio of average nodule mass	0.2	1	5	0.67
Response ratio of total nodule mass	0.2	1	5	0.67
Response ratio of total plant biomass	0.42	1	5	0.55

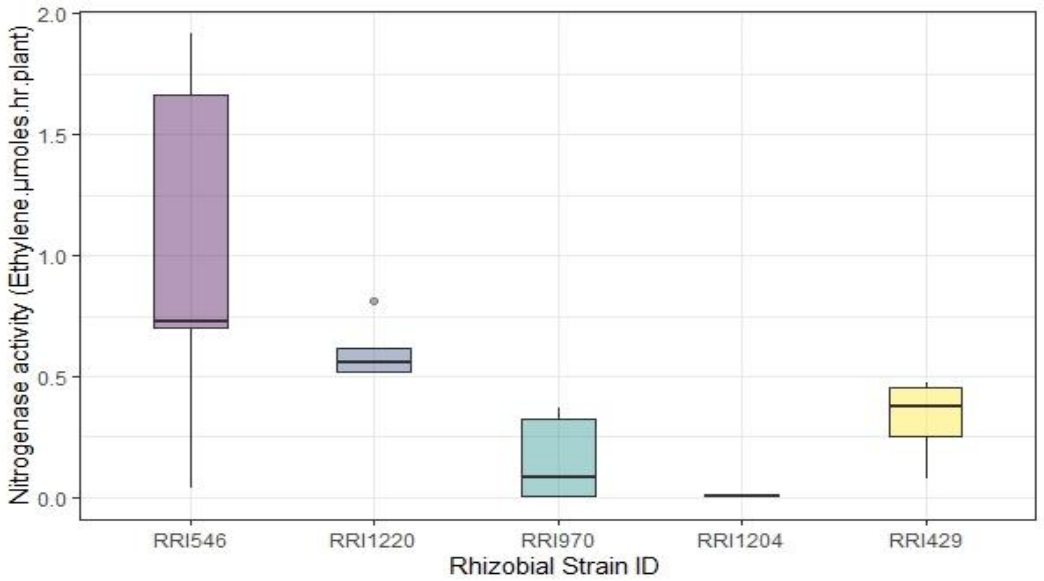

Figure 3. 11: The rate of rhizobial nitrogen fixation in root nodules of pea plants inoculated with single strains of *Rhizobium leguminosarum*. Results shown are for n=6 (biological replicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range.

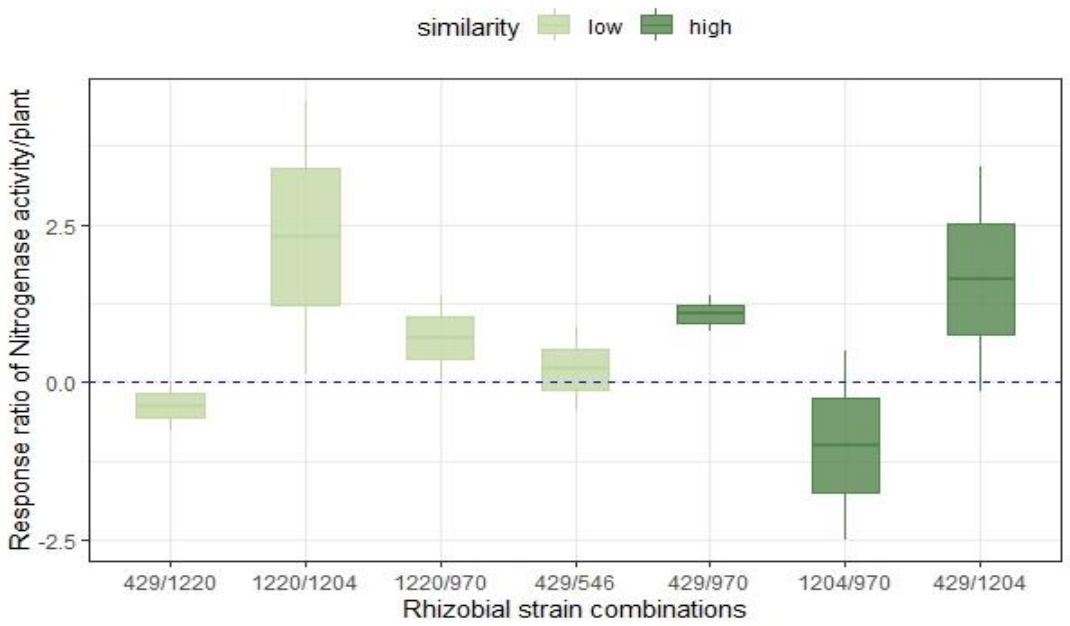

Figure 3. 12: Net co-inoculation effect (measured as response ratios) on the nitrogen fixation efficiency. The zero line depicts no net co-inoculation effect for response variable when the isolate is combined with another isolate. There were no significant differences between two similarity groups (P>0.05, calculated with one-way ANOVA (Type II Wald F tests with Kenward-Roger DF)) as well as among the treatments of the same similarity group (post-hoc analyses).

The co-inoculation effects for the nitrogen fixation rate in pea plants were not significantly affected by the genetic similarity of rhizobial strains (P>0.05, one-way ANOVA-Table 3.4, Figure 3.12). Across both low and high similarity groups, the nitrogenase activity of rhizobial strains was 10%-25% greater in a combination than in individual strain fixation activity (P=0.08, one-tailed t-test, Figures 3.11 and 3.12, Table 3.5). Further, no significant variation of response ratios among strain combinations in each of the similarity group (P>0.05, multiple comparisons) was recorded. The co-inoculation of RRI1204/970 showed 100% reduction of N-fixation compared with single inoculations of RRI970 and RRI1204 as in Figure 3.12.

3.3.3.3 *R. leguminosarum* genetic similarity did not significantly affect field pea plant biomass

The total biomass of pea plants inoculated with different strain combinations did not vary within or across rhizobial genetic similarity groups (P>0.05, Figure 3.13, one-way ANOVA (Type II Wald F tests with Kenward-Roger DF), Table 3.3).

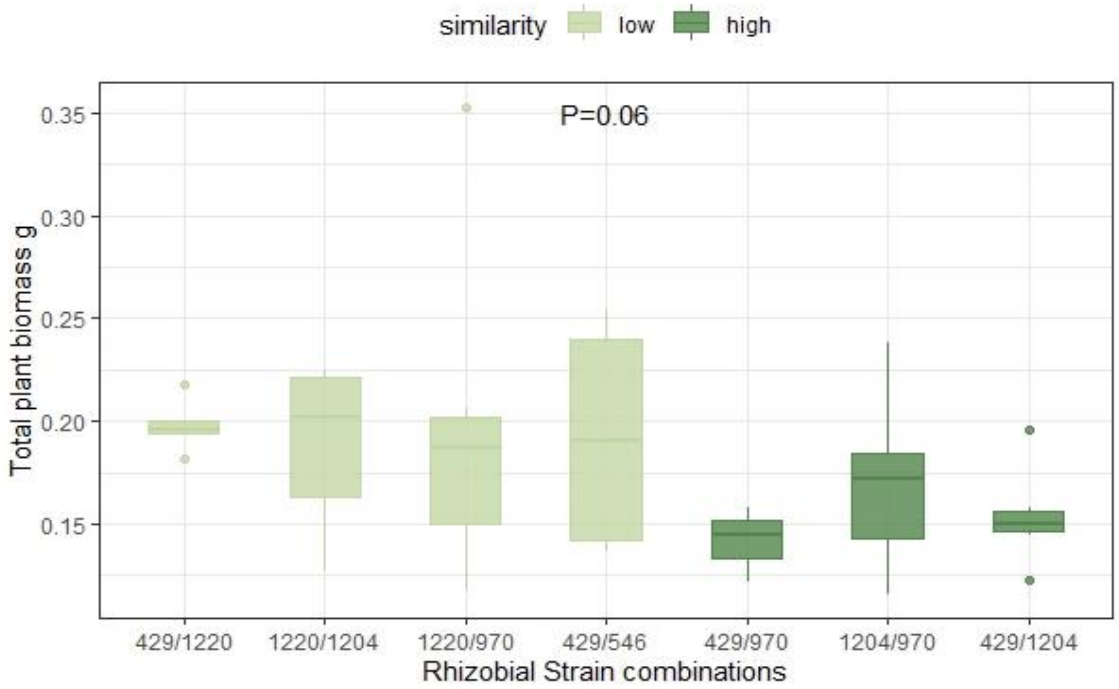

Figure 3. 13: Total plant biomass of pea plants co-inoculated with pairs of *Rhizobium leguminosarum* strains differing in their degree of genetic similarity (based on 16S rDNA sequences). Results shown are for n=6 (biological replicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range. $P_{\text{genetic similarity}}$ was 0.06 (one-way ANOVA (Type II Wald F tests with Kenward-Roger DF))

Further, the net co-inoculation response of plant biomass (Figure 3.15) did not differ between two rhizobial genetic similarity groups (P>0.05, one-way ANOVA (Type II Wald F tests with Kenward-Roger DF), Table 3.4).

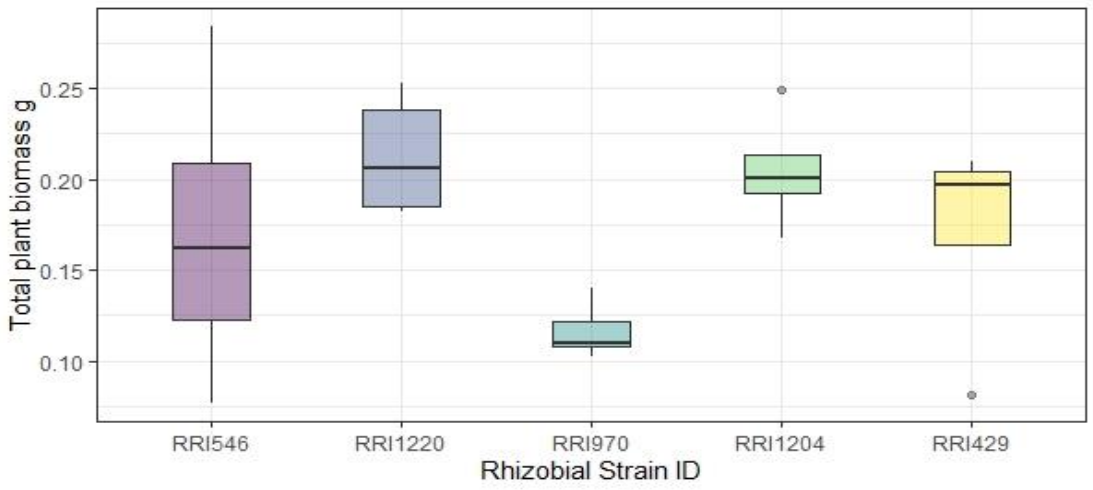

Figure 3. 14: Total plant biomass of pea plants inoculated with single strains of *Rhizobium leguminosarum*. Results shown are for n=6 (biological replicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range.

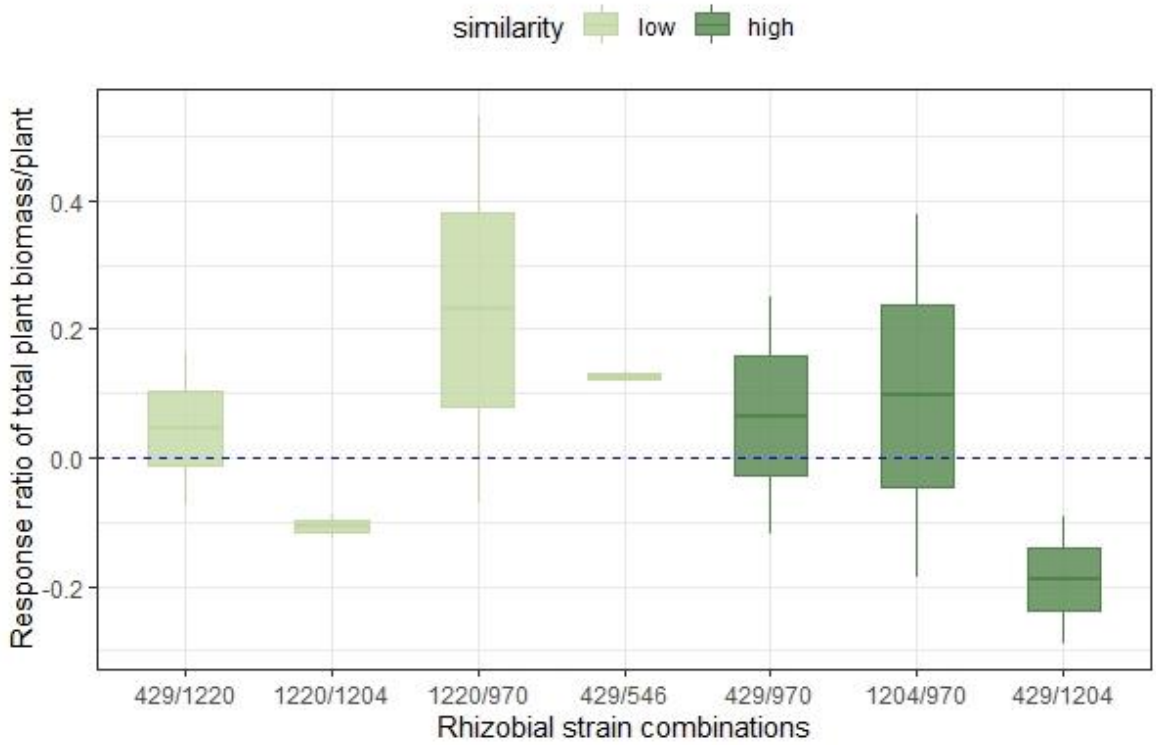

Figure 3. 15: Net co-inoculation effect (measured as response ratios) on the total plant biomass. The zero line depicts no net co-inoculation effect for response variable when the isolate is combined with another isolate. The effect of genetic similarity was not significant at P=0.54 (calculated with one-way ANOVA (Type II Wald F tests with Kenward-Roger DF)). The response did not differ between rhizobial pairs in each similarity group at P>0.05 (multiple comparisons, post-hoc analyses).

The net co-inoculation effect on plant biomass for RRI429/1204 was negative (Figure 3.15) with a decrease in plant biomass by 25% and 9% compared to the single strain performance of RRI1204 and RRI429 respectively (Figure 3.14). In contrast, plants with RRI1220/970 had 70% increased biomass compared to RRI970 on its own. The combination response for plant biomass was 8% lower compared to plants with RRI1220 alone. Co-inoculation of RRI1220/1204 showed 11% decrease in plant biomass compared to RRI1220 inoculation alone (Figure 3.15).

3.3.3.4 Rhizobial genetic similarity was not a significant predictor for assessing average nodule biomass

The rhizobial fitness is expressed by the average nodule biomass obtained in the experiment. I did not observe a significant effect of rhizobial genetic similarity on the average nodule biomass (*P*=0.64, one-way ANOVA (Type II Wald F tests with Kenward-Roger DF), Table 3.3, Figure 3.16). RRI429/970 had >70% bigger nodules (*P*<0.001, Multiple comparisons ('emmeans')) compared to other pairs of higher genetic similarity. Further, RRI429/1220 recorded relatively higher nodule biomass (~44% greater) among less similar rhizobial pairs in the experiment (*P*=0.002, Multiple comparisons ('emmeans'), Figure 3.16).

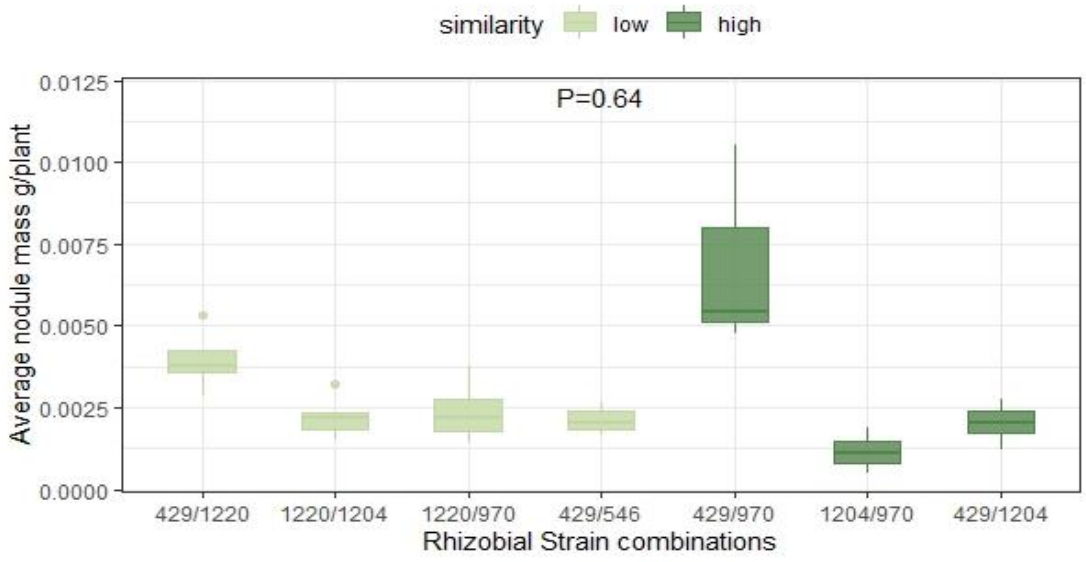

Figure 3. 16: The average nodule mass of pea plants co-inoculated with pairs of *Rhizobium leguminosarum* strains differing in their degree of genetic similarity (based on 16S rDNA sequences). Results shown are for n=6 (biological replicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range. $P_{\text{genetic similarity}}$ was 0.64 (one-way ANOVA (Type II Wald F tests with Kenward-Roger DF)).

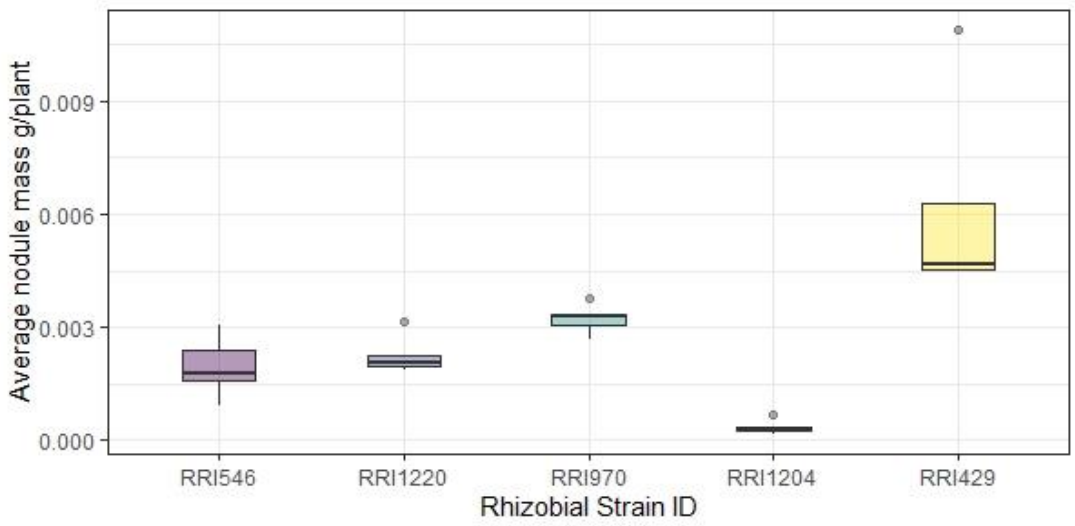

Figure 3. 17: The average nodule mass of pea plants inoculated with single strains of *Rhizobium leguminosarum*. Results shown are for n=6 (biological replicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range.

The net co-inoculation response of average nodule biomass (see Figure 3.18) did not differ between two rhizobial genetic similarity groups (P>0.05, one-way ANOVA (Type II Wald F tests with Kenward-Roger DF), Table 3.4). Further, co-inoculation of RRI429/1220 shows 75% increase in average nodule mass compared to RRI1220 alone where RRI1220 dominates the nodule occupancy by 85% over RRI429 (Table 3.1, Figures 3.17 and 3.18). In a similar way, RRI429/970 shows with 40% and 100% increase of average nodule biomass (Figure 3.18) compared to single inoculations of RRI429 and RRI970 respectively (Figure 3.17), where RRI429 dominates the nodule occupancy by ~96% in the pair-wise inoculation with RRI970 (Table 3.1).

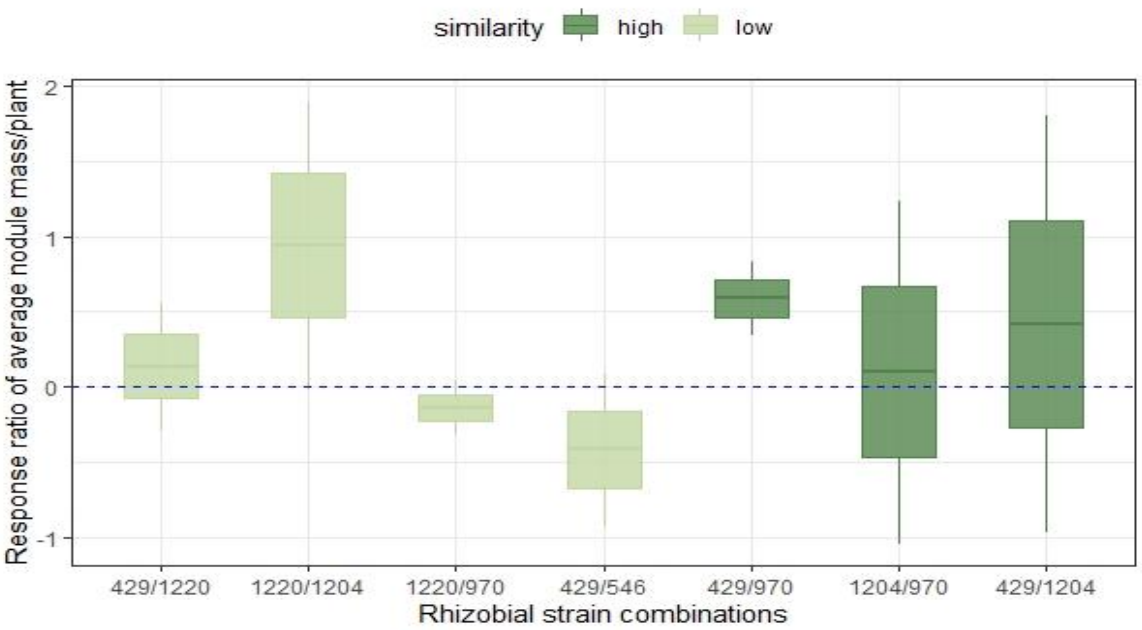

Figure 3. 18: Net co-inoculation effect (measured as response ratios) on the average nodule biomass. The zero line depicts no net co-inoculation effect for response variable when the isolate is combined with another isolate. The effect of genetic similarity was not significant at *P*=0.67 (calculated with one-way ANOVA (Type II Wald F tests with Kenward-Roger DF)). No significant differences among the treatments of the same similarity group (*P*=0.48, post-hoc analyses).

Table 3. 5: Variation in the effectiveness of co-inoculation treatment across both the similarity groups using one sample t-test to test whether the response ratios are greater than zero

Response Ratio	**t**	**df**	***P***
Nodule number/plant	2.48	13	0.01*****
Ethylene µmol/hr/plant	1.43	13	0.08†
Average nodule mass	0.91	13	0.19
Total nodule biomass	2.12	13	0.03*
Total plant biomass	0.61	13	0.28

Signif. codes: '*****' $P<0.05$, '†'$P<0.1$

3.3.3.5 Field pea nodules infected with genetically less similar R. *leguminosarum* strains show higher total nodule biomass compared to the ones with closely related strains

Plant resource allocation with rhizobial colonisation was evaluated using total nodule biomass per plant (Figure 3.19) where the nodules with low similarity rhizobial pairs showed ~33% higher nodule biomass compared to highly similar pairs ($P<0.05$, Table 3.3, Figure 3.19). I did not observe any significant variation of nodule biomass between rhizobial pairs in the low genetic similarity group ($P>0.05$, Multiple comparisons('emmeans')). In the high similarity group, RRI429/970 inoculated plants had significantly higher nodule biomass compared to RRI1204/970 and RRI429/1204 (85% and 80% respectively, $P<0.05$, Multiple comparisons, Figure 3.19).

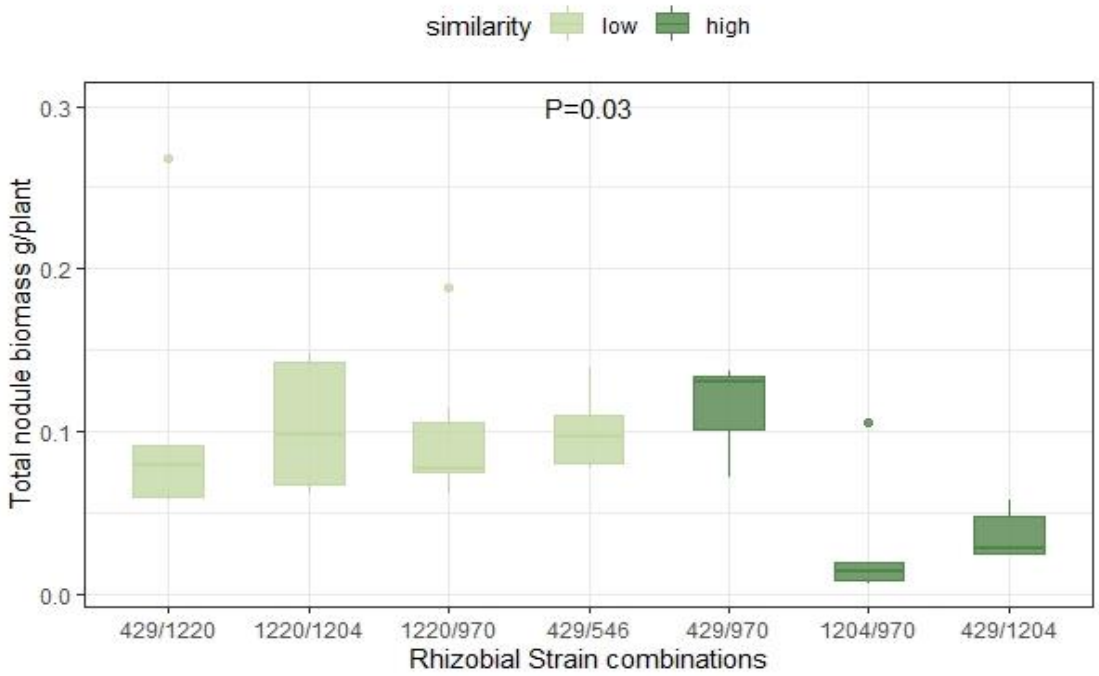

Figure 3. 19: Total nodule biomass of pea plants co-inoculated with pairs of *Rhizobium leguminosarum* strains differing in their degree of genetic similarity (based on 16S rDNA sequences). Results shown are for n=6 (biological replicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range. $P_{\text{genetic similarity}}$ was 0.03 (one-way ANOVA (Type II Wald F tests with Kenward-Roger DF)).

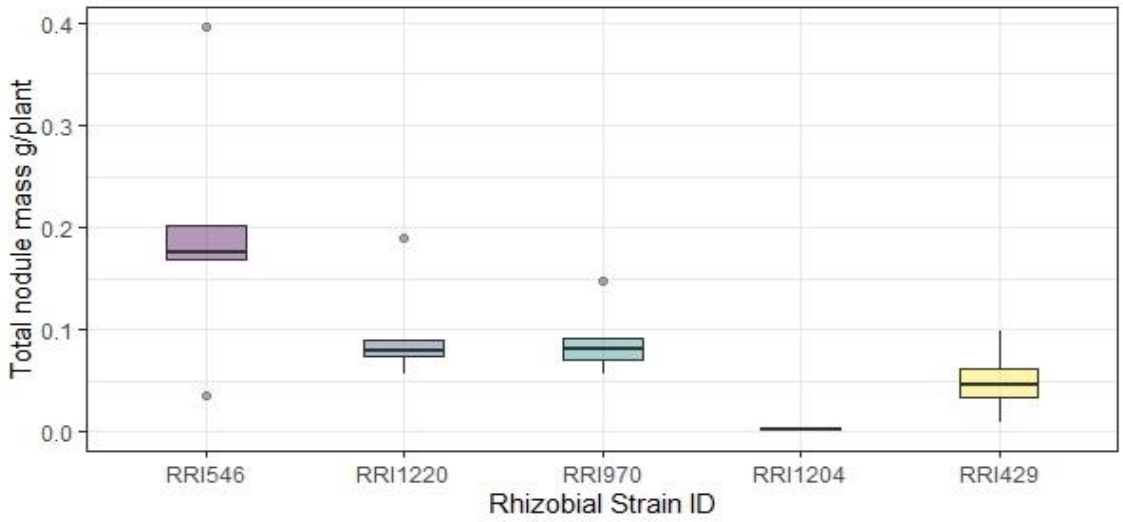

Figure 3. 20: The total nodule mass per pea plant inoculated with single strains of *Rhizobium leguminosarum*. Results shown are for n=6 (biological replicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range.

During the co-inoculation of RRI429 and RRI1204 together, RRI429 was observed in >90% of root nodules (Table 3.2) but total nodule biomass decreased by ~50% in co-inoculation compared to RRI429 on its own (compare Figures 3.20 and 3.21). The co-inoculation effects for the total nodule biomass in pea plants were not significantly affected by the genetic similarity of rhizobial strains (*P*>0.05, one-way ANOVA-Table 3.4, Figure 3.21).

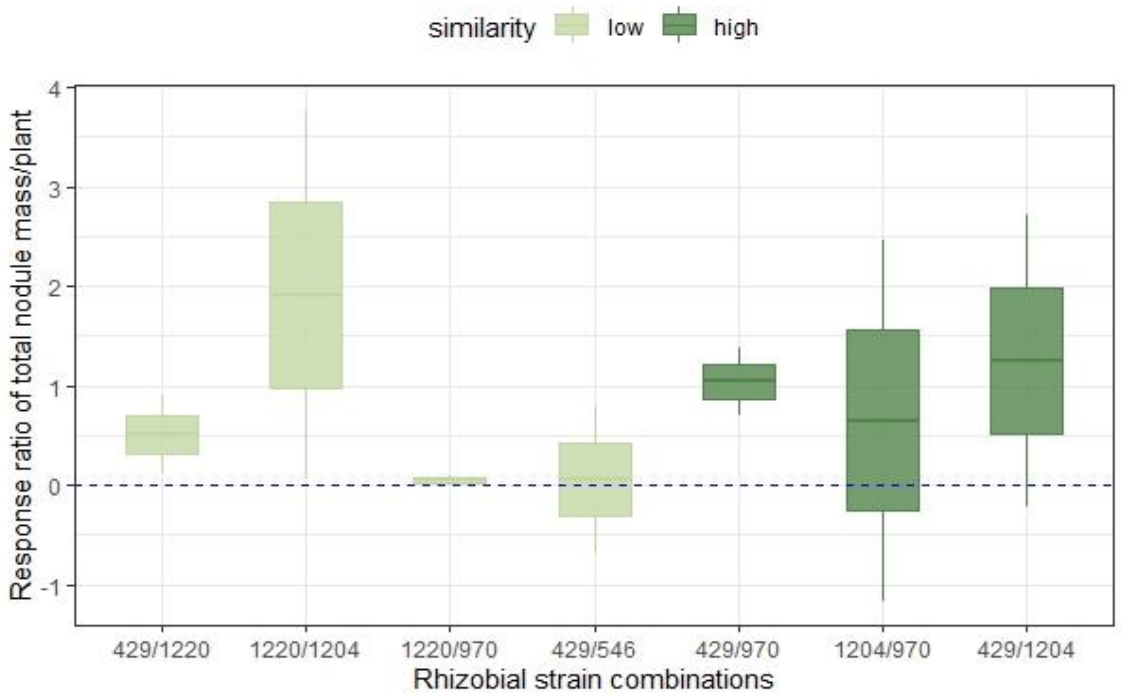

Figure 3. 21: Net co-inoculation effect (measured as response ratios) on the total nodule biomass. The zero line depicts no net co-inoculation effect for response variable when the isolate is combined with another isolate. The effect of genetic similarity was not significant at *P*=0.67 (calculated with ANOVA (Type II Wald F tests with Kenward-Roger DF)). No significant differences among the treatments belonging to same similarity groups (*P*>0.05, post-hoc analyses).

Although the plants inoculated with single strains of RRI1220 and RRI970 had nearly equal total nodule biomasses (mean value of ~0.06 g, Figure 3.20), the pairwise inoculation of RRI1220/RRI970 neither increased nor decreased the nodule mass (Figure 3.21). Regardless the genetic similarity, there was ~8-10% average increase in total nodule biomass response among strain combinations compared to the performance on their own (*P*=0.03, One-sample t-test, Table 3.5).

3.4 Discussion

3.4.1 Mixed nodule infections are infrequent in Field pea–*Rhizobium leguminosarum* symbiosis

To date, this is the first report on evaluating the compatibility of *Rhizobium leguminosarum* strains for producing mixed nodule infections under two different rhizobial genetic similarity groups in field pea root system. My work provides the evidence that pea root nodules with more than one strain of *R. leguminosarum* per nodule in sand-vermiculite substrate were infrequent regardless of strain genetic similarity. Moreover, I observed few infections of multiple strains in single nodules per root system that do not appear to be linked to strain genetic similarity. Having fewer mixed nodule infections may reflect that, in a co-inoculation, the strain with higher competitive nodulation ability surpasses the other resulting in single strain dominated nodules (Svenning et al., 2001).

3.4.2 Strain identity is important in mixtures while inoculation with distantly related rhizobial pairs improves nodulation and N fixation

In line with my hypothesis, when a less similar rhizobial pair was inoculated, the nodule number increased significantly compared to plants with highly similar rhizobial pairs. Moreover, considering the co-inoculation effect, the less similar rhizobial pairs had more nodules than on their own. It is assumed that the colonizing ability of the more competitive rhizobial strain is facilitated by the other strain in the inoculum. This observation is further supported by a previous study of Burton and Martinez (1980) where the formulation of multi-strain rhizobial inoculants enhanced nodulation and N fixation. Further they suggested that the multiple strains could work together to alleviate environmental stress conditions over the single strain inoculants.

Collectively, low similarity rhizobial pairs had significantly higher N fixation (~56%) compared to closely related pairs supporting my prediction that synergistic interactions of rhizobia provide more nitrogen benefits to their host plant. Somasegaran and Bohlool (1990) observed that the multiple strain inoculation fixed ~52% less N compared to the amounts of the single strain treatment of TAL 182 (most effective strain) in the dry bean- *R. leguminosarum* bv. *phaseoli* system. The reasons for reduced N fixation in their study was the competitive dominance of the ineffective strain TAL 1865 which was not compatible with the effective strain TAL 182. The strains RRI1204 and RRI429 in my study produced similar average number of nodules and recorded similar average plant biomass valuesof field pea plants when

inoculated singly. In contrast, N-fixation rates and average nodule biomass values of RRI1204 were 3-fold lower compared to RRI429 despite being more genetically related to each other in their phylogeny (Figure 3.5). Moreover, the plants inoculated with RRI1204 recorded the second highest biomass across other single strain inoculations. This result was unexpected and the reason behind this observation is still unknown.

Some strains such as RRI1220 in my study always outcompeted the other strain in the pair regardless of their genetic similarity. In contrast, RRI970 and RRI1204 showed lower nodule occupancy in most of the pair-wise inoculation treatments except the treatment which included both the isolates together where they co-occurred in the same host (60% and 40% respectively). This observation showed the higher competitiveness of RRI1220 and least competitiveness of RRI1204 and RRI970 among the five RRI strains used in the study. Similarly, May and Bohlool (1983) also observed the variation of nodulation by *R. leguminosarum* combinations due to significant differences in nodulation efficiencies of individual rhizobial strains.

Being efficient N fixers may not always lead to a synergistic N fixing rhizobial interaction between two strains. For example, RRI1220 and RRI429 are genetically less similar and effective nitrogen fixers on their own but they did not appear in the same root system when they were paired. In contrast, RRI1204 and RRI970 despite having high genetic similarity, co-occupied the host plants where RRI1204 has enhanced its fixation efficiency by 66% compared to on its own. Therefore, the co-occurrence of two rhizobial strains in a root system might not be fully dependent on how efficient they are in N fixation. I could also suggest that competitiveness of these strains could be context dependent where each strain combination performs differently though they use the same substrate and environmental conditions.

The genetic similarity of a rhizobial pair did not significantly affect the average nodule biomass or plant biomass response variables in my experiment. Some strain combinations had synergistic increase of their nodule biomass such as RRI429 and RRI970 where the most abundant RRI429 increased its average nodule biomass by 40% and RRI970 by 1-fold compared to their single strain inoculations. In a similar way, RRI546 increased the nodule mass by 9% when it was inoculated with RRI429, but a 60% decrease compared to the average nodule mass of RRI429 on its own. Since average nodule mass could be an indicator for the rhizobial fitness (Friesen, 2012), RRI429 could have increased its fitness when paired with RRI970 in contrast to pairing with RRI546. This evidence adds substantially to my proposed hypothesis that rhizobial strains would not behave the same way as they do on their own in a

co-inoculation. Further work is required to assess whether some strains become more competitive using different mechanisms such as antibiotic production (Kucuk and Cevheri, 2015, Schwinghamer, 1971, Amarger, 1981) or any other inhibitory mechanisms such as releasing toxic proteins to enter the host plant (Lardi et al., 2017, Bladergroen et al., 2003).

Plant resource allocation is a significant factor for rhizobial fitness in legume-rhizobium symbiosis (Kiers et al., 2006) which I measured as total nodule biomass in plant level. Plants co-inoculated with RRI429 and RRI970 had 40% and 100% increased total nodule biomass compared to RRI429 and RRI970 on their own respectively. The co-inoculation of RRI1204/RRI970 increased the overall nodule biomass by 2-fold compared to RRI1204 (the most abundant strain) single inoculation. Further, it is important for the host plant to gain sufficient amounts of fixed N to account for the resources provided for rhizobial symbionts (Kiers et al., 2003). Most of the strain combinations used in my study provided significantly higher N amounts depending on the most abundant strain in the co-inoculation. For example, the co-inoculation with RRI429/970, the percentage increase of N fixation is 100% for RRI429 compared to its single inoculation treatments where as in RRI1204/970, the most occupied strain RRI1204 had increase of N fixation by 66% compared to on its own. Performing the acetylene reductiona assay (ARA) for assessing N fixation rates of nodules had flexibility and ease of use, rapid detectability and low cost (Fulweiler, et al., 2015). However, limitations of this approach have also been noted. For example, physical disturbacnce of nodule-root-soil interfaces with affect detection of accurate N fixation (Unkovich et al., 2008). To overcome this potential limitation, in this experiment nodules were incubated with acetylene over a longer time period (1-2 hours) to ensure consistent and better ethylene peaks. Methods employing δ15N assessment overcome some of the limitations associated with the ARA, and should be considered for future experiments, but were not practically possible here.

3.4.3. Significant findings and proposed future directions

One of the significant findings to emerge from this study is the considerable variation in nodulation and N fixation efficiency in a pea host plant within and among *R. leguminosarum* strain combinations. The findings from this study make several contributions to the current literature by adding more evidence of inter-strain interactions of *R. leguminosarum* in field pea symbiotic system. Although strain competitiveness is a major factor affecting nodulation, there can be other factors such as drought stress, low soil pH (Hirsch, 2010) and the predation of rhizobia by other soil organisms such as protozoa (Danso et al., 1975) that limit root nodulation in field pea. It is recommended that further research should be undertaken with more field rhizobial isolates paired with commercially produced rhizobial inoculants under different

environmental extremes (such as drought). More work on inoculant and resident rhizobial isolates will demonstrate and validate the importance of assessing inter-strain interactions to maximize rhizobial N fixation in cropping fields.

Chapter 4: Drought and rhizobial competition reduced nodulation and N fixation by a commercial *Rhizobium leguminosarum* strain in field pea

4.1 Introduction

The production and application of rhizobial inoculants is a long-established strategy in agriculture for maximizing crop yields (Catroux et al., 2001, Brockwell et al., 1995). A commercial inoculant must cope with both environmental constraints in the field and intense competition imposed by the naturalized or resident rhizobial populations (Denton et al., 2002). These biological and soil constraints demonstrate the importance of using competitive, stress-tolerant, and efficient N fixing rhizobial inoculants, particularly under stressful conditions and in the presence of resident strains competing for space. However, recent work suggests the benefits of coexistence of rhizobial strains in legume symbiosis. For example, Miao et al. (2018) showed that the nodulation efficiency of *Rhizobium etli* can be improved by cohabitation with a resident rhizobium (*Rhizobium fabae*) through quorum sensing. They have found that quorum sensing regulator CinR–mediated gene expression of *R. etli* is promoted by *R. fabae* which enhanced the nodule number of *R. etli*. In my previous work (chapters 2 & 3), I observed that co-inoculating field pea plants with a mixture of genetically less similar *R. leguminosarum* strains resulted in a significant increase in the number of nodules produced. It was proposed that this might reflect reduced competition for space and resources between strains that are more genetically diverse. Moreover, field pea plants grown in red calcarosol soil had limited growth as well as poor rhizobial colonisation and low fixed nitrogen compared to the pea plants grown in black vertosol soil, indicating that the environmental context might also influence the rhizobial growth and function.

Soil water deficit is a major environmental factor limiting successful field pea N symbiosis (Guilioni et al., 2003). Field pea is a winter crop in Australia where the timing of sowing is critical. If the seeds are sown too early they would be exposed to blackspot disease (sometimes called as Ascochyta blight) and if the seeds are sown too late there is likely to be yield loss due to increasing summer temperatures (GRDC, 2017). In the southern regions of Australia (South Australia, Victoria and Tasmania) (GRDC, 2017) and some areas of New South Wales (such as Narrabri, Wagga, Cowra, Nyngan) where field pea is grown, precipitation can be unreliable. For example, in Victoria, the annual precipitation patterns vary from 750 mm to < 500 mm in legume grown fields (Williams et al., 2002). During the long hot summer periods in Australia, it has been found that rhizobial numbers decline as cropping soils become drier causing reduction in effective root nodulation in legume crops (Slattery et al., 2001). It has been

explored that the water stress reduces nitrogen fixation as a result of a decrease in photosynthate supply to nodules (Neo and Layzell, 1997) and/or reduction in the respiratory O_2 supply (Marino et al., 2007) for rhizobia in the nodules. Given the variability of rainfall, and the often-small window before the onset of colder temperatures, the flexibility of a rhizobial inoculant that can persist in suboptimal conditions is of great importance.

The commercial rhizobial inoculants commonly used for inoculating pea are group E (*Rhizobium leguminosarum* SU 303) and group F (*R. leguminosarum* WSM 1455) (Drew et al., 2012). Both the field pea hosts and their rhizobial symbionts prefer well-draining, moist soils within a soil pH ($CaCl_2$) range of 6.0-8.0. Inoculation of legumes with rhizobial inoculants benefits many Australian soils, either because the particular legume has not been grown for many years or becasue various environmental constraint such as drought and salinity limit their abudance and survival (Slattery et al., 2001 and Siddique and Sykes, 1997). Some rhizobia change their morphologies in response to drought which could alter their infectivity and symbiotic potential with the host plant (Zahran, 2010 and Shoushtari and Pepper, 1985). Rhizobia that reside in desiccated soils are well-adapted symbionts (Hussain et al., 2014 and Waldon et al., 1989) and/or 'free-living' under low water potential (Busse and Bottomley, 1989). It has been found that some resident rhizobial isolates are efficient in nodulation and N fixation under arid conditions (Zahran, 1999), although some resident rhizobial communities in Australian cropping soils are poor N fixers due to stressed environmental conditions such as low soil pH and low water availability (Peoples and Baldock, 2001).

Plants play a key role in developing the rhizosphere environment and the nodule spaces for rhizobial partners where it can be assumed that the adverse environmental conditions which affect plant physiology would directly or indirectly affect the rhizobial competition for nodulation (Dowling and Broughton, 1986). For example, Silvente et al. (2012) identified that the metabolic pathways were affected by short-term water limitation in drought sensitive soybean cultivar whereas they observed a reduction in the nodule dry weights in both drought resistant and sensitive cultivars of soybean. I expected that the tolerance of a field pea cultivar to water stress would affect the plant-rhizobial interaction in N symbiosis. Here I have used two pea cultivars having different tolerance levels to water stress and diseases. The cultivar 'Twilight', which is recommended to grow in low rainfall areas of Australia as it has more resistance to drought, and the cultivar 'Wharton', which is well known for its resistance to diseases such as powdery mildew but not particularly for drought (GRDC, 2017). Hence, I assumed that there can be a variation in rhizobial inter-strain interactions in these cultivars due

to different tolerance levels to drought and pathogenic diseases. Further, I expected that the water stress would again significantly affect these rhizobial inter-strain interactions.

This chapter focuses on the efficiency of commercially developed *Rhizobium leguminosarum* inoculant in terms of its ability to persist and compete with resident rhizobia for nodulation and N fixation under low soil moisture conditions. I also looked at whether there is an effect of field pea variety on these inter-strain interactions by using two field pea varieties ('Twilight' and 'Wharton'). As the second aim of this study, I looked at whether the plant responses of nodulation and N fixation efficiency were affected by these inter-strain interactions under low soil moisture availability.

Here I hypothesized that the selective pressure of water availability may influence the interaction between rhizobial strains in root nodules. I suggested that if any drought resistant resident rhizobial strain could build up a synergistic interaction with the commercial inoculant then there can be co-infections of resident and inoculant rhizobia in the same plant root. Moreover, I expected this synergistic interaction to benefit host plants via improved nodulation and N fixation. In contrast, I also hypothesized that if well-adapted resident rhizobia could impose competitive superiority on a commercially introduced strain in a cropping soil, then the persistence and performance of a commercial inoculant will be reduced as it would compete with resident strains for resources under low soil moisture conditions (Howieson and Ballard, 2004). Further, the failure of the commercial strain to colonise the host plant would have a negative impact on the N fixation of the host plant.

As plant physiology plays a key role in improving the rhizosphere environment of rhizobial symbionts, I predicted that the adaptation of field pea hosts to low moisture conditions would influence the inter-strain competition of rhizobial strains in the soil. It was hypothesized that if the field pea cultivar was adapted to low rainfall conditions (cultivar Twilight), it would act as a generalist whereas rhizobial symbionts would nodulate depending on their competitive superiority. Contrarily, the pea cultivar having less drought-resistance (cultivar Wharton) would be severely affected from water stress and lose N symbiotic efficiency with rhizobia as allocating its resources on symbiosis is costly under stressful conditions.

4.2 Materials and Methods

4.2.1 Biological material

Plants

Two field pea cultivars, 'Wharton' (less drought tolerant) and 'Twilight' (drought resistant) were used in the study. The seeds were obtained from Hart Bros Seeds Pty Ltd (Junee Reefs NSW, Australia) and Superior Seeds Co. (Deniliquin, southern NSW, Australia). Surface-sterilization of seeds was done with 10% bleach (Sodium hypochlorite 42 g/L, Sodium Hydroxide 9 g/L and Chlorine 4% w/v (1.9%)) for 10 minutes and followed by six washes of sterile milliQ water.

Rhizobial Strains

Rhizobium leguminosarum bv. *viceae* WSM 1455 (Group F) (GRDC, 2013) was used as the commercial inoculant in this study. The inoculant was obtained in the peat-based formulation (NoduleN™ Peat) from New Edge Microbials, Albury NSW, Australia. Peat-based inoculants provide reliable nodulation scores over freeze-dried forms due to peat's protective formulation against desiccation and exposure to pesticides (Rodriguez-Navarro et al., 1991, Drew et al., 2012). As such, peat-based inoculants are more commonly used in fields compared to freeze-dried forms (GRDC, 2017).

RRI546 and RRI970 were selected as competitor strains. These non-commercial inoculants were obtained from the natural rhizobial collection obtained from Department of Primary Industries, Victoria, Australia. RRI 546- origin: Bhaktapur, east of Kathmandu with average rainfall of 999 mm but only 3-4 months of heavy rainfall. RRI 970- origin: Gallipoli Peninsula Turkey where the average precipitation is about 578 mm. They can be differentiated through ERIC fingerprinting (Refer to Figure 3.6, chapter 3) and they have shown poor capacity for N fixation in the recent experiment completed with sand-plate system (section 3.2, chapter 3).

Soil

Soil was collected from Yarramundi Paddock near Castlereagh Road, Hawkesbury NSW (coordinates: -33.615490, 150.730151). The soil was described as Yarramundi Loam soil which has reddish brown colour with light medium clay texture with a $pH_{(water)}$ of 6.0 and could be ordered as brown vertosols (Isbell, 2016). More details of soil properties can be found in Table 4.1. Soil was not sterilized in the experiment to retain the field nutrient levels. The site was chosen because there has been no history of legume culture and potential hosts at the site and, therefore, no expectation that resident rhizobia would nodulate pea plants and interfere with nodulation and N fixation by the commercial inoculant and competitor strains (RRI546 and RRI970). This was confirmed by planting both the pea cultivars in non-sterile soil in preliminary experiments and there was no evidence of nodulation in either of cultivars.

Table 4. 1: Physical and chemical properties of Yarramundi Loam Soil

Analyte grouping/Analyte	**Units**	
pH ($CaCl_2$)	pH Unit	5.1
pH (water)	pH Unit	5.8
Electrical Conductivity @ 25°C	µS/cm	39
Exchangeable Calcium	meq/100g	2.3
Exchangeable Magnesium	meq/100g	0.6
Exchangeable Potassium	meq/100g	0.8
Exchangeable Sodium	meq/100g	0.1
Cation Exchange Capacity	meq/100g	3.8
Total Alkalinity as $CaCO_3$	mg/kg	27
Bicarbonate Alkalinity as $CaCO_3$	mg/kg	27
Carbonate Alkalinity as $CaCO_3$	mg/kg	<5
Aluminium	mg/kg	4750
Boron	mg/kg	<50
Copper	mg/kg	<5
Iron	mg/kg	6590
Manganese	mg/kg	1820
Molybdenum	mg/kg	<2
Zinc	mg/kg	14
Ammonia as N	mg/kg	<20
Nitrite as N (Sol.)	mg/kg	0.1
Nitrate as N (Sol.)	mg/kg	13.5
Nitrite + Nitrate as N (Sol.)	mg/kg	13.6
Total Kjeldahl Nitrogen as N	mg/kg	660
Total Nitrogen as N	mg/kg	670
Total Phosphorus as P	mg/kg	201
Fluoride Extractable P (Bray)	mg/kg	4.4
Leco Carbon in house	%	0.96

Determining the soil field capacity (FC)

Soil-filled pots were watered from above with sterile milliQ water until free drainage occurred. The pots were covered with aluminium foil to avoid evaporation and kept at 100% saturation for 24 hours. For each soil, the wet weight at 100% FC was measured (Y). Soils were then dried at 105 °C for 24 hours and dry weight (Z) determined. Field water capacity (FWC) was determined using the following equation:

FWC = (Y-Z/ Y) *100

The following calculations were used to achieve the amounts of water (using FWCs) to be added to each pot in well-watered and reduced watering treatments.

Pot weight	25 g
Fresh weight of the soil mixture	400 g
Weight after 100% saturation	500 g
Dry weight of the soil (oven dried at 105°C)	360 g
Amount of water held in fresh soil	(400-360) =40 g
Total amount of water could be held (100% FWC)	(500-360) =140 g
Amount of water at 80% FWC	140*80% =112 g
Amount of water to be added to achieve 80% FWC	112-40=72 g
The total weight of a pot in well-watered treatment	400 g+72 g+25 g **497 g**
Amount of water at 60% FWC	140*60% 84 g
Amount of water to be added to achieve 60% FWC	84-40=44 g
The total weight of a pot in reduced-watering treatment	400g+44g+25 g **469 g**

4.2.2 Inoculum preparation, seed inoculation and planting

The commercial inoculant in peat form was mixed in sterile, cool water (100 g in 2 litres). This selection of inoculant in peat form was based on its higher survival rate in soils compared to freeze dried or granular forms (GRDC, 2017). According to the literature (Berninger et al., 2018), 100g of peat carrier is expected to contain 10^{11} live rhizobial cells. Mixing 100g of WSM1455 peat in 2000ml is expected to have 10^{11} cells. Then inoculating each seed with 1ml from that evenly mixed slurry was expected to contain 10^{8} live cells. The peat inoculant mix was stirred thoroughly for an hour on a magnetic stirrer (Industrial Equipment & Control PTY. LTD, Australia) at maximum speed to disperse evenly in water. The slurry mixture was kept on the stirrer for another 30 min to allow the inoculant adhesive to dissolve (NoduleN™ Peat Instruction guide). The RRI strains were grown in Yeast Mannitol Broth (YMB) (Somasegaran and Halliday, 1982) under 25 °C for 72 hours at 110 rpm in rotary shaker (RATEK, ROWE Scientific Pty Ltd). Both the strains were adjusted to an equal concentration (10^{8} cells/ml) using

absorbance measurements at 600 nm (Plate reader, Agilent technologies, Cary 60 UV-Vis, G6860A). Field pea seeds of both cultivars were surface sterilized with 10% bleach followed by six washes of milliQ water and soaked in respective inocula solutions (1ml per each seed) for 30 min before planting.

Using sterile forceps, the seeds were planted (two seeds per pot) in moistened soils (~400 g) per pot (Height=120 mm and Diameter=70 mm) and they were labelled with inoculant type-cultivar-watering status. After planting the seeds, each pot was supplied with another 1 ml of respective inoculant/mixture of inoculants. Several steps were used to minimise potential for cross contamination. Firstly, the pot soil layer was covered with vermiculite to reduce cross contaminations. Secondly, pots were carefully taken out to weigh and water with sterile miliQ water. Further, each pot was handled individually wearing gloves. Finally, plant growth chambers were bleached and sterile conditions with minimum human/equipment interference throughout the duration of the experiment.

4.2.3 Watering plants and supplying nutrients

Well-watered and water stress treatment

Initially, the pots were maintained at 80% FWC ('well-watered' state) for 3 weeks. Multiple cycles of water stress (see Figure 4.1) were used to make a stress 'memory' in field pea plants (i.e. the structural, genetic, and biochemical modifications in plant tissues to become more resistant to upcoming water-stress conditions) (Tombesi et al., 2018). The expectation was to observe drought symptoms without killing the plant completely. The first cycle of low water treatment with 60% FWC ('water-stressed' state) was applied on four-weeks-old seedlings for one week and followed by a well-watering treatment of 80% FWC for another week. The second drought cycle with 60% FWC was continued for another two weeks followed by a well-watered treatment (80% FWC) until they were harvested. The control plants were watered at 80% FWC throughout these 8-weeks until they were harvested. Fortnightly, N-free nutrient solution (Somasegaran and Hoben, 1985) was added (15 ml/pot) to both well-watered and drought-imposed plants.

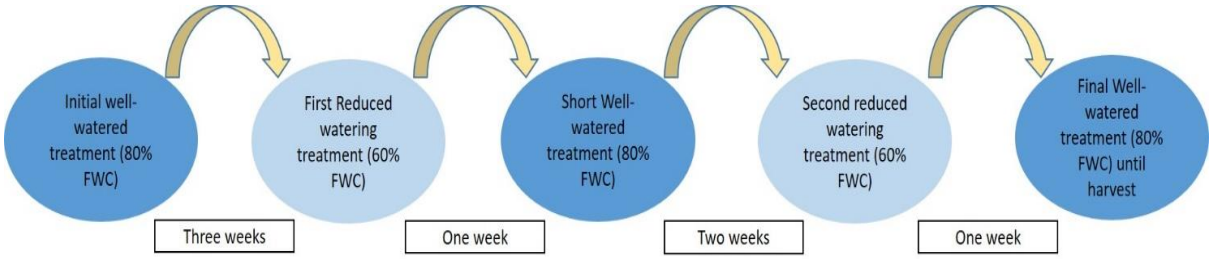

Figure 4. 1: Diagram showing the time durations of well-watered and reduced watering treatments performed in the experiment

Table 4. 2: The three-way factorial experiment design, including the number of replicates for each category.

Treatment/ Inoculation Type	**Watering**			
	Well-watered treatment (80% FWC)		**Drought treatment (60% FWC)**	
	Twilight	**Wharton**	**Twilight**	**Wharton**
WSM1455[1] only	7	7	7	7
Mix of competitors[2] only	7	7	7	7
WSM1455+Competitors	7	7	7	7
Uninoculated	5	5	5	5
WSM1455-Sand	4	4	4	4
Competitors-Sand	4	4	4	4
WSM1455+Competitors - Sand	4	4	4	4
Wheat- Uninoculated	7		7	

1. WSM1455- commercial inoculant *Rhizobium leguminosarum*

2. Competitors- RRI546 and RRI970

A set of plants (48) treated with the commercial inoculant (WSM1455), both competitor strains and mix of WSM1455 and competitors were grown in sand to be used as the inoculated controls for ^{15}N calculations (Table 4.2). Wheat was selected as the un-inoculated control for ^{15}N measurements (see section 4.2.5).

Plants were monitored visually daily for drought symptoms (wilted leaves, stunted growth) and whether there was any pathogen damage (leaf spots). All the plants were supported with bamboo supporters (90 cm) in pots (Figure 4.2). Great care was taken when handling and watering plants to avoid any cross contamination.

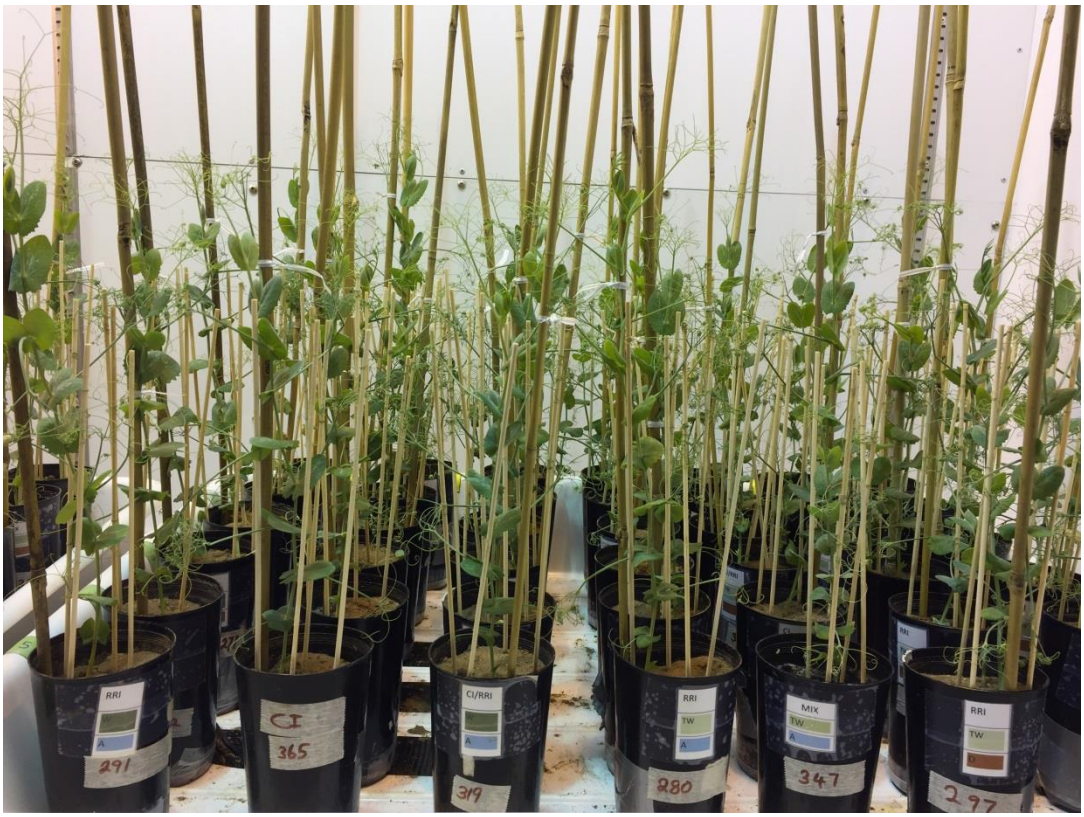

Figure 4. 2: Inoculated and un-inoculated field pea plants under both well-watered and reduced watering treatments arranged in a completely randomized design

4.2.4 Plant harvest, collection of nodules and measuring root and shoot biomass

Plants were harvested when they were 8-weeks old. The roots were washed gently with running water and the total number of nodules counted manually and recorded. All the uninoculated plants in the experiment did not contain any nodules. All the nodules collected from each plant were placed in glass vials (12 ml), and an acetylene reduction assay performed as previously described (chapter 3, Materials and methods, Section 3.2.4.1). Nodules were then removed from vials, weighed and then stored at -20 °C until DNA extraction. Roots and shoots were separated, labelled and dried at 70 °C for three days and dry weights recorded. Dried roots and shoots were stored in desiccators on silica medium until further analysis.

4.2.5 Analysis of whole plant level nitrogen fixation using natural abundance of ^{15}N in shoot tissues

Dried shoots were finely ground using Fast Prep®-24 tissue and cell homogenizer (M.P. Bio medicals, California, USA). Powdered samples were weighed (4.5-5.5 mg, microbalance XP6, Mettler-Toledo Ltd., Port Melbourne VIC 3207, Australia) into tin capsules, crimped and analysed for ^{15}N by UC Davis Stable Isotope facility, CA, USA. Care was taken when using tools and workspaces to avoid contaminations from ^{15}N labelled work. The percentage of nitrogen derived from the atmosphere (%Ndfa) was determined for shoot samples using the following formula (Polania et al., 2016);

$$\%Ndfa = \frac{\delta^{15}N \text{ of the reference plant} - \delta^{15}N \text{ of test legume}}{\delta^{15}N \text{ reference plant} - \beta} \times 100$$

Reference plant= non-fixing (non-legume Wheat)

(Note: I had uninoculated pea plants with no visible nodules (assumed no fixation) but wheat was taken as the reference plant since it was 100% confirmed with no N fixation). $\beta = \delta^{15}N$ of the nodulated pea plant solely dependent on fixed N (grown in sand) for N requirement.

4.2.6 Extraction of bacterial DNA and strain identification

Root nodule samples obtained after the acetylene reduction assay (stored at -20°C) were crushed and lysed as individual nodules (five nodules/plant) to obtain rhizobial DNA using Isolate II Plant DNA kit BIO 52070 (Bioline Pty Ltd, Australia). The DNA was eluted using elution buffer (60 µl) and the concentrations were measured using NanoDrop 2000 Micro-volume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Delaware USA) by using 1.5-2 µl of eluted DNA at the absorbance of 260 nm.

Identification of the rhizobial strain/s in single nodule DNA extracts was done using ERIC PCR and agarose electrophoresis as previously described (Chapter 3- Materials and methods, section 3.2.1). The resulting banding patterns were compared with the bands obtained for the original strains of WSM1455, RRI546 and RRI970 (supplementary Figure S4-1, Appendix 3).

4.2.7 Statistical Analysis

All the data were analysed using R version 3.5.1 (R Development Core Team, 2016). Data from 95 plants (both single and multi-strain inoculated plants) were included in the analyses. All the

variables were square-root transformed, tested to check whether they follow a normal distribution using 'Shapiro-Wilk test' in R 3.5.1 (Shapiro & Wilk, 1965) and residuals were checked using normal QQ plots.

Three independent variables; rhizobial inoculation type (WSM1455, competitors and mixed inoculation), field pea cultivar (Twilight and Wharton) and watering status (well-watered or water-stressed) were used to evaluate the plant responses observed in the study. Linear models were constructed to look at the effect of these independent variables on nodule number, nodule level nitrogen fixation (nitrogenase activity per nodule), plant level nitrogen fixation (using ^{15}N data), total nitrogen of the plant, plant biomass, total and average nodule biomass. Total nodule biomass was considered as the total plant investment in nodule formation to facilitate rhizobial fitness. *P* values were determined using three-way ANOVA- 'car package' (Fox & Weisberg, 2019).

The function 'emmeans' in package 'emmeans' (Lenth et al., 2019) was used to calculate Tukey's Honestly Significant Differences for comparing the different levels within a factor (multiple comparisons). For example, the means of the three rhizobial inoculation types were compared with each other ([WSM1455 v. Competitors] [WSM1455 v. Mixed] [Competitors v. Mixed]) under each combination of watering and cultivar conditions. The plots were constructed using the package 'ggplot2' (Wickham, 2016) using the significant main effects and their interactions observed for plant responses.

The relationship between plant biomass and fixed amount of nitrogen was assessed by fitting simple linear regression and analysing the correlation coefficient. The percentage nodule occupancy of the commercial inoculant and the competitor strains in a mixed inoculation treatment were calculated and plotted against cultivar and watering conditions using 'ggplot2' (Wickham, 2016).

The Relative Interaction Index (RII) was calculated to represent the ratio of the net WSM1455 occupancy of a competitive interaction with competitor strains (numerator) relative to the facilitative interaction (denominator); this can be expressed as

$$\mathbf{RII} = \frac{\mathbf{\%Nodule\ Occupancy\ of\ WSM1455 - \%Nodule\ Occupancy\ of\ Competitors}}{\mathbf{\%Nodule\ Occupancy\ of\ WSM1455 + \%Nodule\ Occupancy\ of\ Competitors}}$$

RII is symmetrical around zero and a negative value of RII represents negative competition and positive value is a prediction for a facilitative or dominance of WSM1455 in the mixed infected

root. One sample t-test was performed to check whether the RII of WSM1455 was significantly higher compared to competitors across both the cultivars and watering conditions. ANOVA (Type II Wald F tests with Kenward-Roger df) in 'car' package (Fox & Weisberg, 2019) was performed to determine whether there are significant effects of cultivar and watering on the presence of WSM1455, competitors and or mixed nodule infections.

4.3 Results

4.3.1 Nodule occupancy by commercial (WSM 1455) and competitor strains differed among cultivar and watering treatments

The percentage nodule occupancy by the commercial inoculant WSM1455 was significantly greater compared to the competitors in mixed inoculation treatments across both pea cultivars under well-watered and drought conditions (P<0.01, One sample t-test, t = 3.295, df = 24, Figure 4.3).

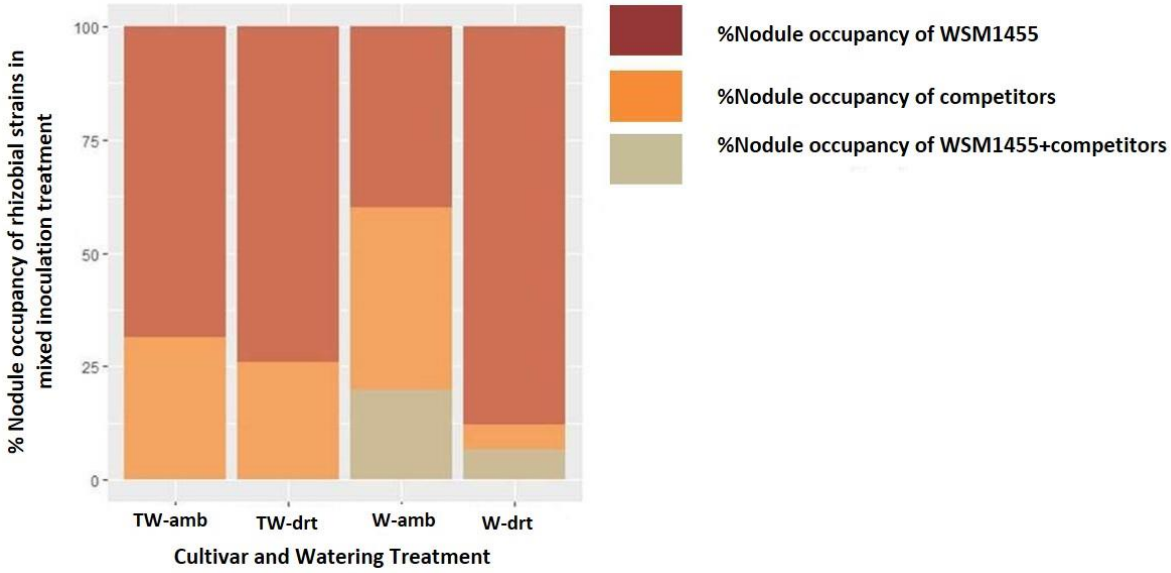

Figure 4. 3: Percentage nodule occupancies of the commercial inoculant (WSM1455), competitors and both in Mixed inoculation treatments performed in cultivar Twilight (TW) and Wharton (W) under well-watered (amb) and drought (drt) conditions. Percentages are calculated from the mean values of each treatment (n=4-6 plants and 20-30 nodules per treatment)

The probability of observing either the inoculant (WSM1455) or competitors or both together within a nodule depending on the plant cultivar and watering status of the plant was evaluated by fitting logistic regression. The cultivar Wharton facilitated mixed nodule infections (WSM1455+competitors) compared to cultivar Twilight (P=0.01, ANOVA-Type II), Table 4.3) and there was not any significant effect of watering treatment (P=0.3) on the mixed nodule infection. There was also not interaction of cultivar and watering on mixed nodule infection (P=0.99). The percentage nodule occupancy of the competitor strain RRI546 was significantly higher compared to RRI970 (P<0.05, Two-sample t-test, t = 25.5, df = 29). Most of the mixed infected nodules contained RRI546 and WSM1455 (seven mixed infected nodules with WSM1455+RRI546 and two mixed nodules with RRI970+WSM1455).

Table 4. 3:Summary of ANOVA (Type II Wald F tests with Kenward-Roger DF) showing the effects of cultivar and watering on WSM1455, competitors or mixed nodule infections

Effect	WSM1455 infection			Competitor infection		Mixed nodule infection	
	Df	**LR Chisq**	***P***	**LR Chisq**	***P***	**LR Chisq**	***P***
Cultivar	1	0.32	0.57	3.11	0.08†	6.28	0.01*
Watering	1	1.9	0.17	2.79	0.09†	1.08	0.3
Cultivar: Watering	1	2.27	0.13	0	1	0	0.99

***P*<0.05* and *P*<0.01†**

Nodule occupancy by the WSM1455 was not significantly different between cultivars nor watering conditions. The percentage nodule occupancy of competitors was marginally higher in well-watered conditions (~38%) compared to water stress conditions (~6%) in plants of cultivar Wharton whereas I did not observe such variation in cv. Twilight (Figure 4.3 and Table 4.3).

4.3.2 Total number of nodules in pea plants varied between rhizobial inoculation types, pea cultivars, and reduced watering conditions

The total nodule number per individual plants ranged from 0 to ~35 and was significantly affected by the type of rhizobial treatment ($P<0.05$, Three-way ANOVA, Table 4.4). Competitor strains on their own produced ~50% higher number of nodules compared to the plants infected with WSM 1455 ($P<0.05$, Multiple comparison [Competitors v. WSM1455], Figure 4.4).

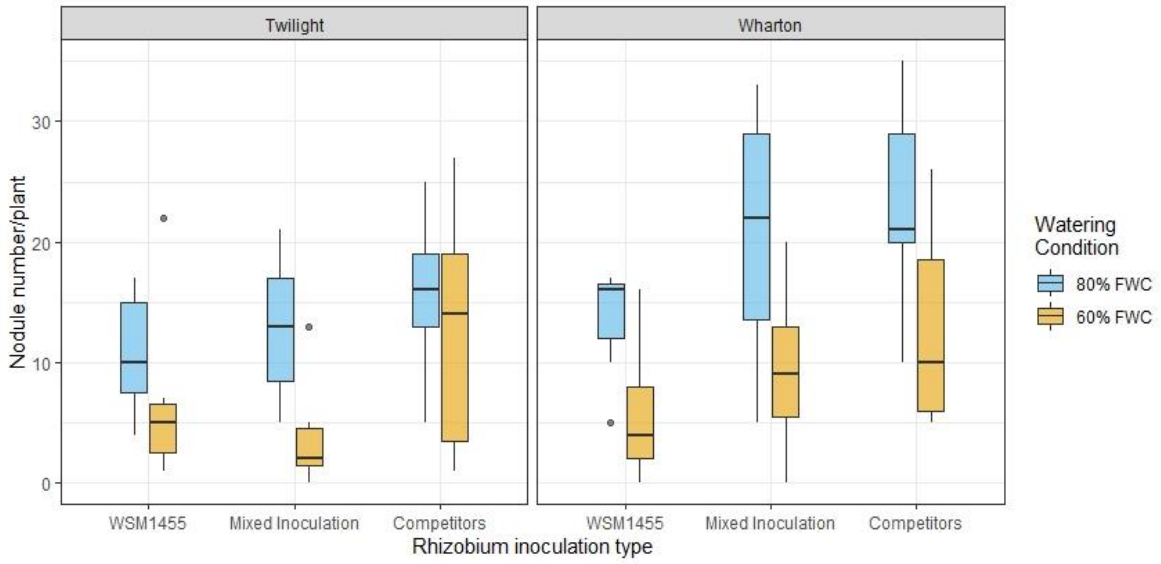

Figure 4. 4: Nodulation response of field pea hosts inoculated with three rhizobial treatments (WSM1455=Commercial inoculant of Rhizobium leguminosarum, Competitors = R. leguminosarum RRI546 and RRI970 & Mixed=mix inoculation of the WSM1455 and competitors) (n=7 each) under well- watered (80% FWC) and drought (60% FWC) conditions in two different cultivars; Twilight and Wharton. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range**.**

The mixed inoculation of WSM1455 and competitors together resulted in an intermediate number of nodules, 27% less than competitors on its own and 22% greater than WSM1455 on its own (Figure 4.4, Multiple comparison [Competitors v. Mixed] and [WSM1455 v. Mixed], $P>0.05$). Reduced watering (60% FWC) resulted in a significant reduction in nodulation (~48%) compared to well-watered (80% FWC) treatments ($P<0.01$, Three-way ANOVA, Table 4.4, Figure 4.4). Cultivar Wharton had a marginally higher (~38%) number of nodules ($P=0.05$, Three-way ANOVA, Figure 4.4, Table 4.4) compared to cultivar Twilight across all the inoculation treatments. The interactive effects among cultivars, isolates and watering status on nodule numbers were not statistically significant.

Table 4. 4: Summary of three-way analysis of variance (ANOVA- Type II Wald F tests with Kenward-Roger df) between rhizobial isolate, cultivar and watering condition on nodule number, nodule and plant level N fixation, total shoot N, average nodule biomass and total plant biomass

Source of variation	Nodule Number/plant			Nitrogenase activity/nodule		Amount of fixed N (using ($\delta^{15}N$) Shoot		Total Shoot N		Total nodule biomass /plant		Average Nodule biomass		Plant biomass	
	DF	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Isolate	2,72	5.73	<0.01**	0.24	<0.01**	4.61	0.01 *	5.89	<0.01**	0.63	0.536	2.27	<0.01**	2.74	0.07†
Cultivar	1,72	4.08	0.05†	0.21	0.65	13.81	<0.01**	6.75	0.01 *	1.97	0.165	0.67	0.42	23.5	<0.01**
Watering	1,72	26.89	<0.01**	1.97	0.16	5.45	0.02*	15.19	<0.01**	8.88	<0.01**	1.92	0.17	20.1	<0.01**
Isolate x Cultivar	2,72	1.15	0.32	0.61	0.54	0.49	0.61	0.44	0.65	0.35	0.708	1.22	0.3	0.48	0.62
Isolate x Watering	2,72	0.61	0.54	0.17	0.84	0.21	0.81	1.64	0.20	0.3	0.74	2.71	0.07†	3.7	0.03*
Watering x Cultivar	1,72	0.66	0.42	1.53	0.21	0.25	0.61	0.02*	0.87	0.04*	0.85	0.11	0.74	0.87	0.35
Isolate x Cultivar x Watering	2,72	0.7	0.704	2.24	0.11	0.03	0.97	0.39	0.68	1.21	0.31	1.21	0.3	0.004	0.99

***P*<0.01 **, *P*<0.05* , *P*<0.1†**

4.3.3 The average nodule mass was lower when WSM1455 and competitors were inoculated together

Although the mixed inoculations contained higher percentages of WSM1455 in nodules (Figure 4.3), the average size of the nodules produced by WSM1455 in the presence of competitors was significantly smaller (~86% reduction in size) compared to nodules with WSM1455 alone (Multiple comparisons [WSM1455 v. Mixed], $P < 0.01$, Figure 4.5). The nodules of WSM1455 and competitor inoculated plants of cultivar Twilight showed significant reduction in size (~50-75%) compared to the nodules of competitors and WSM1455 on their own under reduced watering treatment ($P_{Isolate*watering}=0.07$, Three-way ANOVA, Table 4.4) (Multiple comparisons [Competitors v. Mixed], $P<0.05$, Figure 4.5). The interactive effects of cultivar and watering on average nodule biomass were not statistically significant ($P>0.05$, Three-way ANOVA, Table 4.4, Figure 4.5).

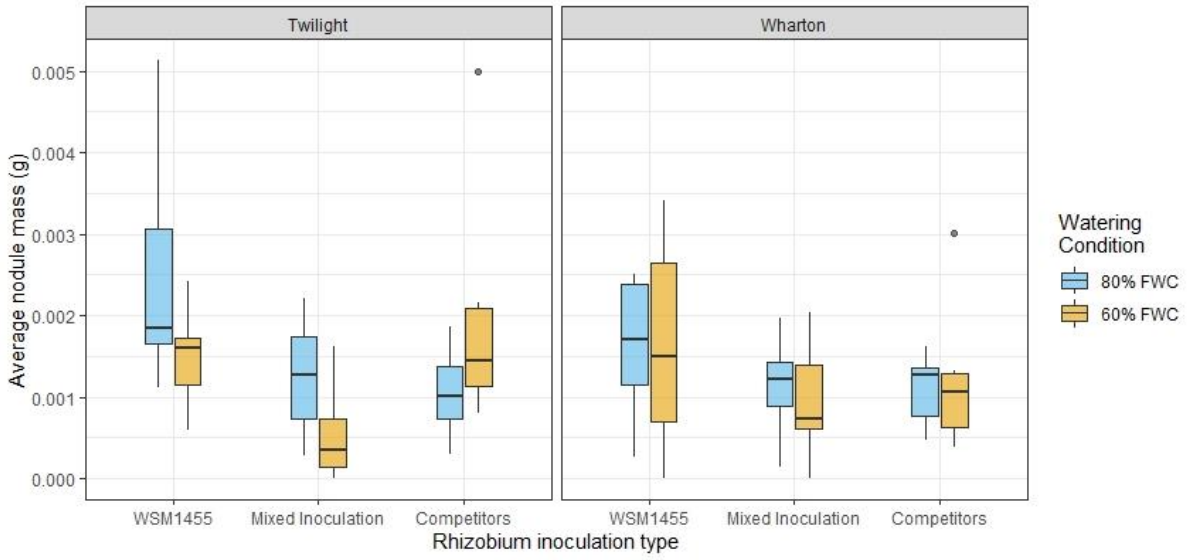

Figure 4. 5: Average nodule biomass (g) of field pea hosts inoculated with three rhizobial treatments (WSM1455=Commercial inoculant of *Rhizobium leguminosarum*, Competitors = *R. leguminosarum* RRI546 and RRI970 & Mixed=mix inoculation of the WSM1455 and competitors) (n=7 each) under well-watered (80% FWC) and drought (60%FWC) conditions in two different cultivars; Twilight and Wharton. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

4.3.4 Competitors provided less nitrogen benefits to their host plants compared to WSM1455

The type of rhizobial isolate significantly affected the amount of nitrogen fixed in the host plant (P<0.01, Three-way ANOVA, Table 4.4). Although the competitors could produce higher numbers of nodules (~50% higher than WSM1455), the amounts of nitrogen fixed in nodules were significantly less (~55% less) than the amounts provided by WSM1455 on its own (Multiple comparison [Competitors v. WSM1455], P<0.05, Figure 4.6). In a mixed infection with competitors and WSM1455, the rate of N-fixation in nodules was not as high as in single inoculation of WSM1455 (~64% higher) (P=0.03, Multiple comparison [Mixed v. WSM1455]). Neither cultivar nor drought had significant effects on nodule level N fixation (P=0.65**,** P=0.16, Three-way ANOVA, Table 4.4).

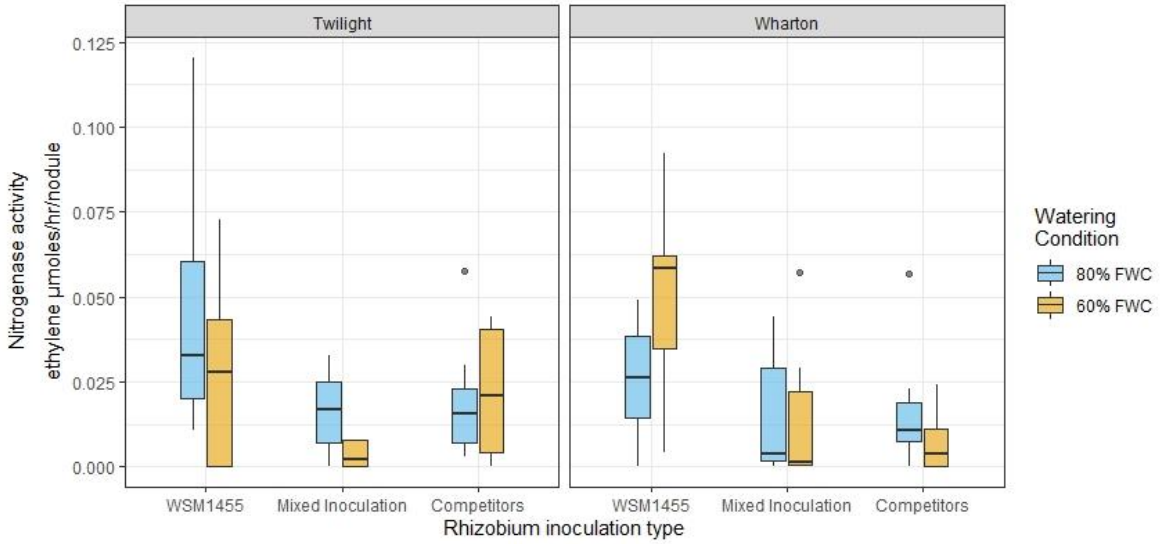

Figure 4. 6: Nodule level nitrogen fixation efficiency of field pea hosts inoculated with three rhizobial treatments (WSM1455=Commercial inoculant of *Rhizobium leguminosarum*, Competitors = *R. leguminosarum* RRI546 and RRI970 & Mixed=mix inoculation of the WSM1455 and competitors) (n=7 each) under well-watered (80% FWC) and drought (60% FWC) conditions in two different cultivars, Twilight and Wharton. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

Three-way interactive effect of isolate, cultivar and watering was not statistically significant (P=0.08, Three-way ANOVA, Table 4.4). WSM1455 infected Twilight nodules showed higher fixation rate (~60% greater) alone under well-watered conditions compared to nodules with competitors or mixed rhizobial treatment (P<0.05, [WSM1455 v. Competitors] and [WSM1455 v. Mixed], Multiple comparisons). Cultivar Wharton showed the similar response but only in reduced watering condition (P<0.01, Multiple comparisons, P=0.03 [WSM1455 v. Competitors] and [WSM1455 v. Mixed] respectively).

The total amount of fixed N in plant shoot varied between the two cultivars (Figure 4.7, $P<0.01$, Three-way ANOVA, Table 4.4) where Wharton showed ~30% higher N fixation compared to Twilight ($P<0.05$, Multiple comparisons). Water stress negatively affected total amount of fixed N ($P=0.02$, Three-way ANOVA, Table 4.4) averaged over the levels of cultivars and isolates. Any significant interactions of isolate, cultivar and watering status on the fixed N amounts in plant shoot tissues were not encountered ($P=0.97$, Three-way ANOVA, Table 4.4).

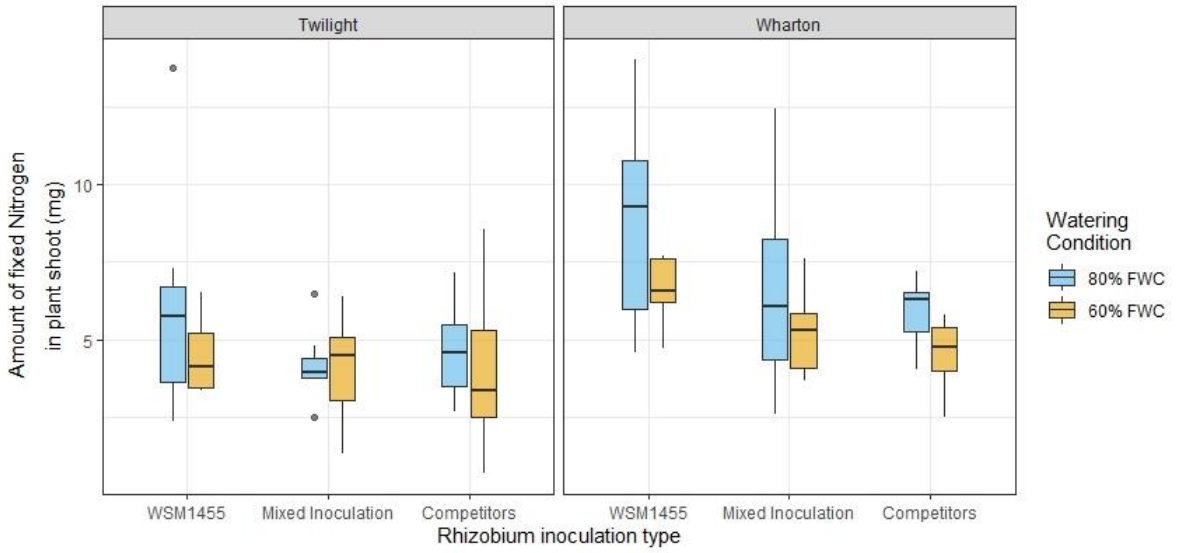

Figure 4. 7: The total amount of fixed N in shoot tissues inoculated with three rhizobial treatments (WSM1455=Commercial inoculant of *Rhizobium leguminosarum*, Competitors = *R. leguminosarum* RRI546 and RRI970 & Mixed=mix inoculation of the WSM1455 and competitors) (n=7 each) under well- watered (80% FWC) and drought (60% FWC) conditions in two different cultivars, Twilight and Wharton. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

Competitor strains alone had significantly less N fixed in the nodules compared to WSM1455 alone ($P=0.04$, [Competitors v. WSM1455], Multiple comparisons). There was also a marginal increase of fixed N in WSM1455 alone compared to mixed inoculation ($P=0.06$, [WSM1455 v. Mixed], Multiple comparisons, Figure 4.7) when averaged over two cultivars.

4.3.5 Total nodule biomass of field pea hosts did not vary among different rhizobial infections

The effects of rhizobial isolate type and cultivar on plant investment on total nodule biomass were not significant under well-watered conditions ($P>0.05$, Three-way ANOVA, Figure 4.8 and Table 4.4). Water stress caused significant reduction (~50%) in total nodule biomass (mean

= 0.0094 g/plant, $P<0.05$, Three-way ANOVA, Table 4.4, Figure 4.8) compared to well-watered treatment (mean = 0.018 g/plant-averaged over the levels of cultivars and isolates).

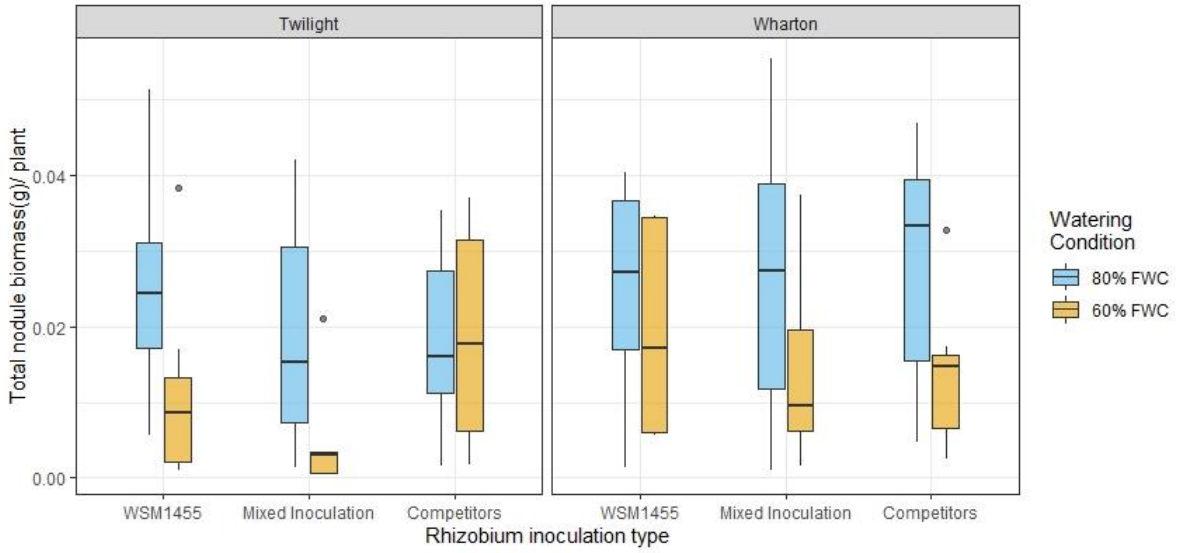

Figure 4. 8: Total nodule biomass of field pea plants belong to cultivars Twilight and Wharton with three rhizobial treatments (WSM1455=Commercial inoculant of *Rhizobium leguminosarum*, Competitors = *R. leguminosarum* RRI546 and RRI970 & Mixed=mix inoculation of the WSM1455 and competitors) (n=7 each) under well-watered (80% FWC) and drought (60% FWC) conditions in two different cultivars; Twilight and Wharton. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

There was no strong evidence that the rhizobial inoculation type, cultivar and the watering status interacted with each other for a significant effect on total nodule biomass response ($P>0.05$, Three-way ANOVA, Table 4.4).

4.3.6 Commercial inoculant alone contributed to higher total N content of the host plant compared to competitors

The commercial *R. leguminosarum* inoculant WSM1455 inoculated plants had higher (~35%) shoot nitrogen content compared to competitors (Multiple comparisons [WSM1455 v. competitors], $P<0.05$, Figure 4.9) or to the mixed infected roots (~44% greater, Multiple comparisons [WSM1455 v. Mixed], $P<0.05$, Figure 4.9) averaged over two cultivars.

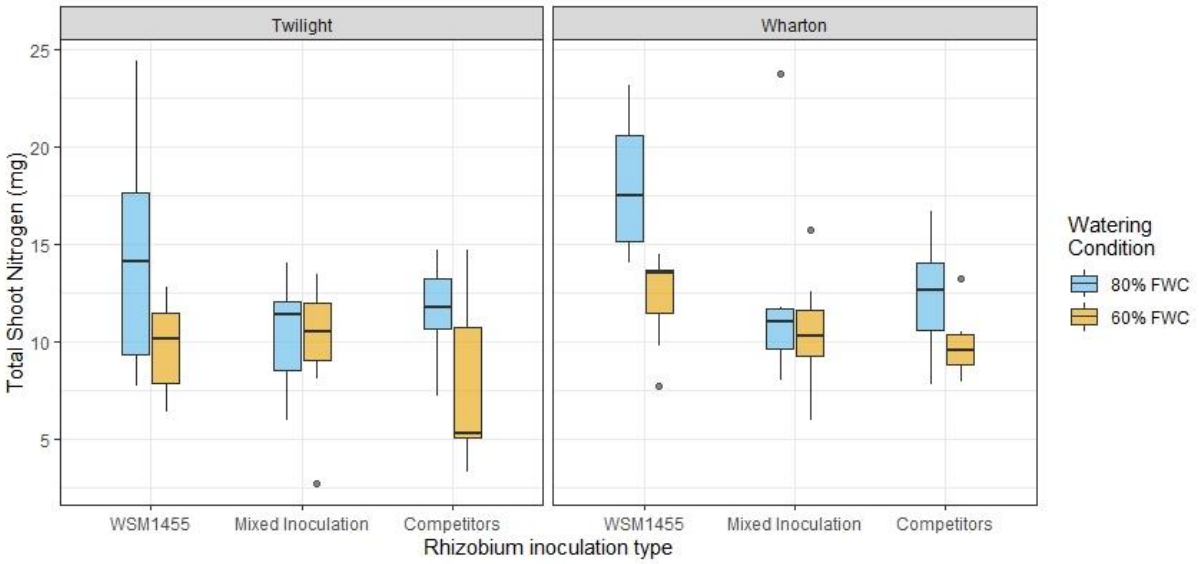

Figure 4. 9: Total plant (shoot) nitrogen content of field pea hosts inoculated with three rhizobial treatments (WSM1455=Commercial inoculant of *Rhizobium leguminosarum*, Competitors = *R. leguminosarum* RRI546 and RRI970 & Mixed=mix inoculation of the WSM1455 and competitors) (n=7 each) under well-watered (80% FWC) and drought (60% FWC) conditions in two different cultivars, Twilight and Wharton. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

Water stress significantly reduced (~32%) the total nitrogen content of the host plants (P<0.01, Three-way ANOVA, Figure 4.9, Table 4.4) and cultivar Wharton showed ~18% more shoot nitrogen compared to cultivar Twilight (P=0.02, [Wharton v. Twilight], Multiple comparisons, averaged over the isolates). I did not find significant evidence to show that isolates, cultivar or watering conditions interacted with each other to cause significant effects on total plant nitrogen (P>0.05, three-way ANOVA, Table 4.4).

4.3.7 Presence of competitors reduced commercial inoculant's ability of increasing host plant biomass

My analyses showed a significant interaction between the type of rhizobial isolate and watering status on variation in plant biomass (P=0.03, Three-way ANOVA, Table 4.4). WSM1455 on its own increased plant biomass (~35%) compared to its performance in the presence of competitors (P<0.05 [WSM1455 v. Mixed], Multiple comparisons) under well-watered condition. However, under the drought conditions, I did not see any significant effect of the isolate on the plant biomass (P>0.05 in each case [WSM1455 v. Competitors], [WSM1455 v. Mixed] and [Competitors v. Mixed], Figure 4.10).

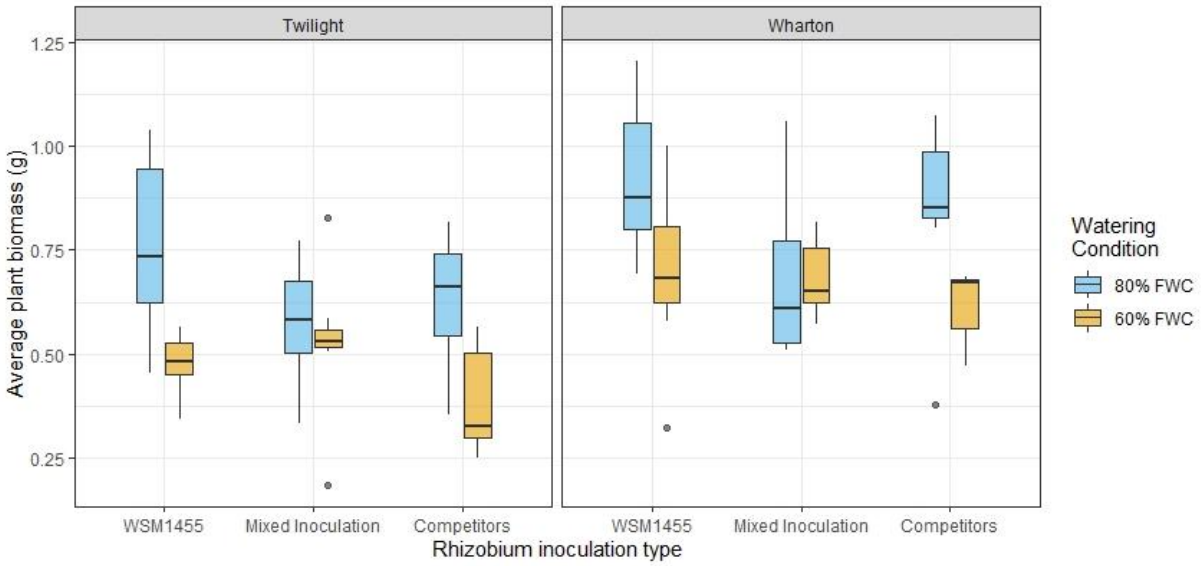

Figure 4. 10: Average total biomass of field pea plants inoculated with three rhizobial treatments (WSM1455=Commercial inoculant of *Rhizobium leguminosarum*, Competitors = *R. leguminosarum* RRI546 and RRI970 & Mixed=mix inoculation of the WSM1455 and competitors) (n=7 each) under well- watered (80% FWC) and drought (60% FWC) conditions in two different cultivars, Twilight and Wharton. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

When the results are averaged over the levels of isolates and cultivars, water stress negatively affected (~32% reduction) plant biomass (P<0.01, Three-way ANOVA, Table 4.4) compared to well-watered treatment. Further multiple comparison analysis showed that the cultivar 'Wharton' had higher (~32%) plant biomass compared to 'Twilight' (P<0.01 [Twilight v. Wharton]).

4.3.8 Atmospheric N fixation and plant biomass was significantly correlated under well-watered and water stressed conditions

Atmospherically fixed N significantly correlated with plant biomass in both Twilight and Wharton cultivars in both well-watered conditions (r= 0.64 and P<0.01 (t = 5.2904, df = 40)) and in water- stressed conditions (r= 0.685 and P<0.01 (t = 5.9416, df = 40)). There was a significant linear relationship between the total amount of fixed nitrogen and plant biomass (Figure 4.11).

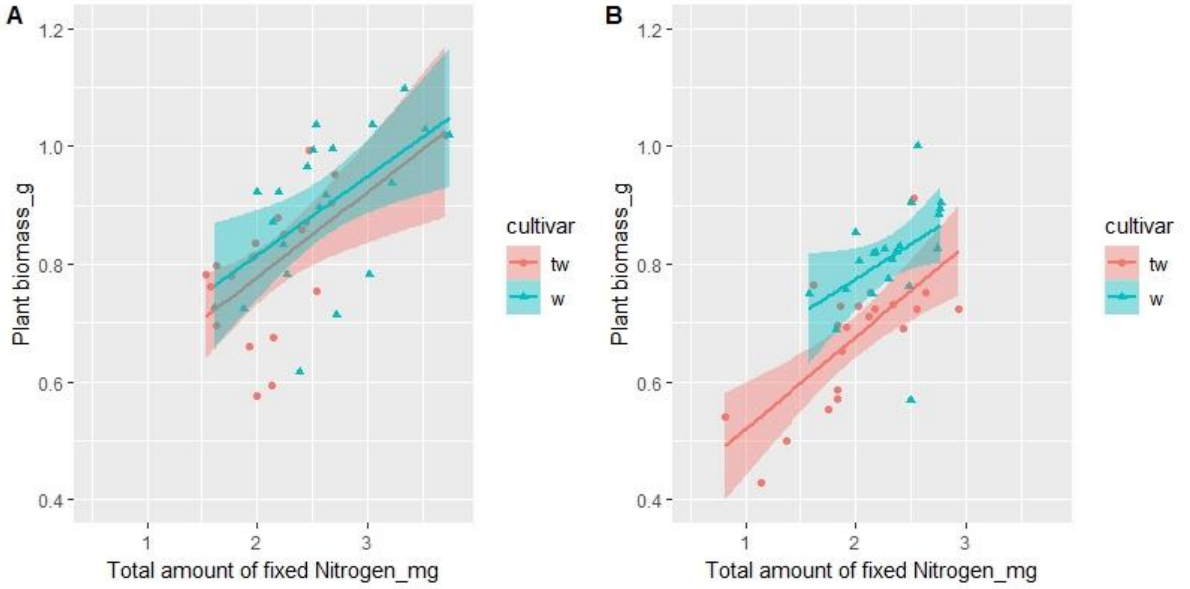

Figure 4. 11: Correlation between total amount of fixed nitrogen and plant biomass. The relationship was evaluated taking the plant biomass as a function of two pea cultivars ('Wharton' and 'Twilight') and the total amount of fixed nitrogen under (A) well-watered (80% FWC) and (B) water-stressed (60% FWC) conditions, fitted with simple linear regression. The shaded band in each of the regression line is a pointwise 95% confidence interval on the fitted values (the line). This confidence interval contains the true, population, regression line with 0.95 probability.

The cultivar Wharton fixed more N (~42% and ~31% respectively) under both well-watered and reduced watering conditions (averaged over the levels of inoculation types) compared to cultivar Twilight (P=0.02, Three-way ANOVA and Multiple comparisons, Figure 4.11, Table 4.4). However, a reduction was observed in plant biomass in response to fixed N under water stressed conditions (P<0.01, Figure 4.11 (B)). Further, cultivar Wharton had higher total plant biomass (~25% and ~43% respectively) under both well-watered and reduced watering conditions compared to cultivar Twilight (P<0.01, Three-way ANOVA and Multiple comparisons, Figure 4.11, Table 4.4). The total biomass of control plants did not significantly vary between the two cultivars under well-watered or reduced watering treatments (P>0.05, Two-way ANOVA, Table S4-1, Appendix 4).

4.4. Discussion

The current study explored the inter strain interactions between *Rhizobium leguminosarum* WSM1455 commercial inoculant and competitor strains of *R. leguminosarum* (RRI546 and RRI970) in field pea N symbiotic system. It was also determined whether soil water and pea cultivar had any significant effect on these interactions under controlled environmental conditions. Overall, the commercial inoculant WSM1455 alone produced larger, fewer nodules with significantly higher N fixation regardless of plant cultivar or watering condition. On the other hand, competitors alone produced relatively smaller, but more, nodules which gave less N benefits to the host plant regardless of cultivars or watering treatment. Further, the nodules produced by WSM1455 in the presence of competitors (mixed inoculation) were smaller and fixed less N compared to on its own and had less effect on plant nitrogen and enhancing plant biomass. The total plant investment in nodule biomass did not vary between rhizobial inoculation type and cultivars. Atmospherically fixed nitrogen significantly correlated with enhancing the plant biomass in both the cultivars.

4.4.1 The commercial inoculant WSM1455 dominates field pea nodules when inoculated together with competitors

My results show that WSM1455 was more frequently recovered in nodules in mixed inoculation treatment than competitors regardless of cultivar or watering status. WSM1455 could be more competitive compared to RRI546 or RRI970 when entering plant roots and formation of nodules. Having WSM1455 in peat formulation might have also caused better survival of cells compared to residents, which were in yeast mannitol growth media when inoculated. There was also a limitation to measure the number of viable cells in peat inocula of the commercial inoculant where I had to calculate approximately the number of cells per gram of peat using the instruction manual given in the peat inoculum guide. However, a similar finding of WSM1455 inoculant was observed by Ballard et al. (2018) where the nodulation efficiency was 80% greater compared to non-inoculated controls in Kaspa field pea plants. According to Souza et al. (2015), the microbial competition around the plant root is mostly driven by intense molecular signalling and rhizodeposition by the plant (deposition of nutrients in the plant rhizosphere). This complex signalling mechanism might have caused the rhizobium-legume interaction to be specific. Initiation of rhizobial-legume symbiosis depends on both plant secretion of flavonoids to induce *nod* genes in rhizobia and the activity of lipo-chitin oligosaccharides (synthesized in bacteria) (Hungria and Stacey, 1997). Therefore, there can be many reasons for WSM1455

being more abundant in nodules such as efficient molecular signalling, improved motility towards the host root (Capdevila et al., 2004), better attachment to root tissues (Buell and Anderson, 1992), higher growth rate inside nodules (Browne et al., 2009) and stress resistance capacity compared to other rhizosphere bacteria (Martinez et al., 2009). Future work is required to test whether WSM1455 has a competitive advantage according to the above-mentioned traits.

4.4.2 Competitor rhizobia produce more nodules with low average nodule biomass in single inoculations compared to WSM1455 in field pea hosts

Competitor strains were more capable of producing higher numbers of nodules in pea hosts compared to WSM1455 alone. I assumed that the competitor strains might be having higher nodule forming ability than WSM1455 in single inoculation treatments and also they might have adaptations to survive in soils during seed inoculations (Denton et al., 2002). As described by Amarger (1981), resident rhizobial populations might show competitive ability for nodule formation similar to effective inoculants. My work further demonstrated that the average nodule biomass of competitors (single inoculations) and the nodules formed during mixed infections were significantly less than the nodule biomass produced by WSM1455 on its own. I suggest that resource allocation by plants to individual nodules may be greater for WSM1455 colonised nodules. Partly in line with my findings, Singleton and Stockinger (1983) found that both ineffective residents and effective inoculants colonise the same legume host but carbon resources are favourably provided to the effective nodules.

The average nodule biomass was lower in mixed inoculation treatments compared to WSM1455 alone. It might be that nodules with competitor strains have lower biomass compared to the nodules with WSM1455 and caused a reduction the average nodule biomass in mixed inoculation treatment. Another possibility is that the inoculant could be left with low energy for N fixation for the host plant while competing with competitors for getting more nodule occupancy. Host plants would not invest their carbon resources for inefficient nodules leading to reduction of the nodule biomass compared to effective N fixing nodules. This predicted scenario is further discussed in the host nitrogen benefits section 4.4.3. I observed that the reduced watering caused a reduction in nodule number across all the inoculations and cultivars. Water stress of the plants might have disrupted the functions inside nodules such as carbon metabolism and protein synthesis leading to low cell growth and drying out (Gil-Quintana et al., 2013). Reduced watering status could also influence rhizobial proliferation in

soil where rhizobia would be less likely to survive and function under low water availability (Zahran, 1999).

4.4.3 The commercial inoculant alone was more efficient in providing N benefits to host plants compared to its presence with competitors

In this study, nitrogen fixation efficiency of WSM1455 alone was greater compared to competitor strains. In contrast, the nodules occupied by WSM1455 in the mixed infection treatment had significantly lower N fixation efficiency compared to WSM1455 inoculation alone. This was obtained with both nitrogenase activity observed in nodules (acetylene reduction assay) and plant level N fixation ($\delta^{15}N$ dilution) data. Heath and Tiffin (2007) suggested a mechanism of strain antagonism which may have occurred in the experiments conducted here. When an effective rhizobium strain is coupled with an ineffective but competitive strain, then the effective strain would face competitive pressure and perform less well compared to on its own. Similarly, the reduced performance of WSM1455 in a mixed inoculation might be due to the competitive pressure of competitor strains used in the study. I observed the same results with total plant nitrogen accumulation where the plants inoculated with WSM1455 had more N in plant tissues compared to the plants inoculated with either the competitor strains or WSM1455 and competitor strains together. This agreed with the proposed hypotheses that the efficiency of a commercial inoculant would be negatively affected if it had to compete with ineffective N fixing strains resulting in reduced N benefits to the legume host. As described by Kiers et al. (2006), the host plant would not favour rhizobia fixing less N in nodules by cutting off the oxygen supply to less/non fixing nodules. They determined soybean plant sanction response to rhizobia fixing at three rates ~1%, 17%, 33%, and 50% of potential fixation where the reproductive fitness of rhizobia recovered as ~37%, 40%, 61%, and 77% respectively. This might be a possible reason for getting less average nodule biomass in mixed inoculation treatment though they are dominated by WSM1455.

There was a significant decrease of N fixation and total N accumulation under water stressed conditions in both the field pea cultivars probably due to the reduction of nodulation. Serraj et al. (1999) describes that all the processes associated with rhizobium-legume symbiosis would be affected by soil water deficit. Inhibition of the nitrogenase enzyme which catalyses N fixation reaction (Minchin, 1997) under water limited conditions was associated with decreased activities of several other carbon metabolising enzymes in nodules as well (Sheoran et al., 1988). Observing that the cultivar Wharton had more fixed N compared to cultivar Twilight

suggests that the legume cultivar might play an important role in N symbiosis. This is further supported by the findings of Hardarson and Atkins (2003) where they observed a significant variation in N fixation among twenty common bean (*Phaseolus vulgaris*) cultivars having very inefficient cultivars as well as better fixing cultivars. One reason for cultivar Wharton to be more efficient in N fixation might be an effective rhizobium interactions compared to cultivar Twilight (Awonaike et al., 1992). However, my work did not provide sufficient evidence to confirm the interactive effect of rhizobium inoculation type and cultivar on N fixation response.

4.4.4 Presence of competitor rhizobia lowers the ability of WSM1455 to enhance plant biomass

My results showed that under well-watered conditions (defined by 80% FWC) the plants inoculated with WSM1455 significantly increased plant biomass by ~35% compared to competitors infected plants. In a mixed inoculation with competitors, however, WSM1455 was less efficient, having low average plant biomass similar to resident rhizobia infected plants. I could also observe a significant interaction between water stress (60% FWC) and the type of inoculation where the differences in plant biomass in response to isolate type became non-significant under water stress. As described by Zahran (1999), the rhizobial strains reduce their ability to fix nitrogen under water-stressed conditions except for a drought-tolerant strain. Therefore, I suggest that under reduced watering conditions, the commercial inoculant WSM1455 was also less effective in N symbiosis similar to ineffective competitor strains resulting in reduced biomass of host plants due to low N supplement (Weyens et al., 2009). Water stress might also disrupt plant metabolism and mineral nutrition resulting in decreased plant growth (Leung and Bonomley, 1994).

The total plant investment into nodule formation was estimated as the total nodule biomass per plant. I observed that rhizobial inoculation type had less significant effects due to individual plant variation within treatments. Water stress had a significant negative effect on nodule biomass possibly be due to impairing the N fixation process and reduction of plant resource expenditure on nodule formation (Athar and Johnson, 1996).

4.4.5 Cultivar Wharton had mixed nodules infected with competitor strains and WSM1455

Mixed nodule infections were observed with WSM1455 and RRI546 in cultivar Wharton but not in cultivar ‘Twilight’. Similarly, Liang et al. (2019) observed that the mixed infections of

two different rhizobial strains (*Rhizobium leguminosarum* Norway and *Mesorhizobium loti*) depend on the host (*Lotus burttii*) ability of encountering different strains. The rhizobial entry to a nodule involves either an infection thread or an epidermal/transcellular infection which leads to two or more rhizobia in one nodule. Therefore, I suggest that a similar mechanism might be leading RRI546 and WSM1455 to form mixed nodules, but more experimental evidence is required to confirm the actual mechanism. Further, my results showed that the cultivar Wharton had more mixed nodule infections compared to cultivar 'Twilight'. One possibility is that cultivar Wharton might have the ability of attracting multiple rhizobial strains allowing mixed nodule infections (Awonaike et al., 1992). Another possibility is that cultivar Wharton is bigger, fixing more carbon and presumably exuding more C to the rhizosphere. Perhaps they could be purely more attractive because there is a stronger C gradient from the rhizosphere to surrounding soil to promote proliferation in the rhizosphere. However, more research looking at the interaction of field pea cultivars with different rhizobial inocula is required to understand the association of two rhizobial strains in single nodules.

4.4.6 Conclusions and future directions

In conclusion, the commercial inoculant WSM1455 on its own was an effective symbiont giving host plant more N benefits and enhancing plant biomass. In line with my hypotheses, the competitor rhizobial strains (RRI546 and RRI970) were less efficient in N fixation. The presence of competitor strains reduced the WSM1455's efficiency of N fixation in the host plant suggesting the negative effects of inter-strain competition between them. Having higher numbers of the inoculant in a mixed infection treatment raises a potential question whether the host plants could try to sanction the ineffective rhizobia at the point of entry in the presence of an effective inoculant. If this is the case, then it would be important to look at the mechanisms driving the inoculant to be less efficient in N fixation during mixed infections. Inoculant-resident competition is an enduring challenge due to the complex nature of their interactions (Triplett and Sadowsky, 1992). More research is required to determine the possible mechanisms/adaptations of residents causing competitive pressure on commercial rhizobial inoculants in the field.

Considerably more work is required to determine the effects of prolonged drought on rhizobial inoculant and resident's efficiency in field pea N symbiosis. There can also be other environmental constraints such as low soil pH having significant effects on altering the rhizobial interactions in field conditions. Thus, continued efforts are required to address the

effects of host-environment drivers on inter-strain interactions and rhizobial fitness. It has been suggested that there is a large diversity of non-rhizobial endophytic bacteria that co-exist with rhizobia in nodules (De Meyer et al., 2015) though their role is not clearly defined. There can be a possibility that these non-rhizobial endophytes directly or indirectly affect the rhizobial inter-strain interactions since they share the nodule space and host carbon resources (Lu et al., 2017). Hence my future work will also be focused on the diversity of these non-rhizobial endophytes in different rhizobial inoculation treatments and whether these communities would vary under water stressed conditions.

Chapter 5: Drought and rhizobial inoculation altered communities of non-rhizobial endophytes in field pea nodules

5.1 Introduction

Rhizobia are the dominant nitrogen (N) fixing bacteria in legume root nodules (Wielbo et al., 2007). With the advancement of isolation methods and molecular technologies, there is increasing evidence that nodules can also be co-infected other non-rhizobial endophyte (NRE) bacteria (Martínez-Hidalgo and Hirsch, 2017). Some endophytic bacteria (other than rhizobia) that colonise root nodules may be effective partners for N-fixing rhizobia (Sturz et al., 2000) and also some NRE bacteria in nodules may possess plant growth promoting genes to facilitate legume growth (Moulin et al., 2001). For example, *Burkholderia* (Burkholderiaceae) strains discovered in *Phaseolus vulgaris* (common bean) (Talbi et al., 2010) and *Vigna unguiculata* (cowpea) not only induced root nodulation but promoted the growth of host plants (Soares et al., 2014). Further, some exhibit the potential to reduce disease via biocontrol activity against pathogens. For example, the *Burkholderia cepacia* JBK9 showed potential as a biocontrol agent by producing pyrrolnitrin against plant fungal pathogens (Jung et al., 2018). Root nodules may also be niches for highly diverse non-rhizobial bacteria that neither nodulate nor are directly involved in the N-fixation process. The nodule endophyte *Gluconacetobacter diazotrophicus* is used as a commercial inoculant in soybean fields along with rhizobial inoculants (Reis and Teixeira, 2015). *Gluconacetobacter diazotrophicus* does not possess N fixing or nodulating genes but the presence of this NRE resulted in an increase in yields of soybean inoculated with rhizobia. Although the specific mechanisms of these endophytic bacteria are unknown, it is suggested that they might facilitate the functioning of rhizobia in nodules.

Invasion via the rhizobial infection thread is one strategy for non-rhizobial endophytes to enter the host plant. Pandya et al. (2013) examined that two non-rhizobial bacteria, *Pseudomonas fluorescens* IAM 12022 and *Klebsiella pneumoniae* ssp. *ozaenae*, entered nodules of *Vigna radiata* via the infection thread of *Ensifer adhaerens* (rhizobia). This phenomenon raises an important question whether these non-rhizobial endophytes co-infect the nodules with rhizobial inhabitants and share the same nodule space, nutrients (photosynthetic carbon from the host) and respiratory oxygen in the nodule (White et al., 2007) while opportunistically colonising this habitat (Zhang et al., 2018).

In some cases, host plants may favour the presence and activity of these nodule endophytes, especially for alleviating environmental extremes such as drought in combination with rhizobial symbionts (Figueiredo et al., 2008). Further, when plants are in a stressed condition (such as drought), the phytohormone balance is found to be mediated by endophytic bacteria in plant tissues (Plazinski and Rolfe, 1985). Egamberdieva et al., (2017) described that root-associted beneficial microbes can produce phytohormones that influence plant growth and function under abiotic stress conditions. Mediating the phytohormone balance in plants under stressed condition not only improves plant growth but also enhance mineral nutrition and plant resistance to pathogen attack (Kudoyarova et al., 2019). Under nutrient deprived conditions, some NRE produce phenolic compounds in legume roots, which can improve flavonoid secretion by roots and attract N-fixing bacteria to form nodules (Juszczuk et al., 2004). Survival and selection strategies of these NRE are still unanswered questions in many legume symbiotic systems. Water deficit may reduce nodulation (Hungria and Vargas, 2000, Athar and Johnson, 1996). The legumes grown in water-limited soils of Australia showed high genetic and phenotypic diversity of nodule-associated symbionts (Barrett et al. 2015, Thrall et al. 2000). Little is known about how these NRE communities interact with each other and with rhizobia during environmental stress (Vuong et al., 2017). Examining the NRE diversity associated with different rhizobial strains/combinations under environmental extremes provides a promising opportunity to enhance understanding of rhizobial interactions and their effects on the composition and structure of endophyte communities in root nodules.

My previous work evaluating inter-strain interactions of *Rhizobium leguminosarum* strains in field pea N-nutrition involved the co-inoculation of pea hosts with closely and distantly related rhizobial strains to determine how genetic similarity of rhizobial pairs (*Rhizobium leguminosarum*) affected nodulation and N fixation efficiency (chapters 2 and 3). In addition to DNA from *R. leguminosarum*, there was a high frequency of DNA from NRE bacteria in nodules of plants for which closely related rhizobial pairs were inoculated (~50-80% of NRE sequences per plant). It was hypothesised that stronger competition between closely related *R. leguminosarum* strains may have resulted in more niche space (Steele et al., 2011) available for NREs, compared with the outcome of weaker competition between distantly related strains.

Many Australian soils are poor in nutrients and have low water availability in general (Thrall et al., 2011). Prolonged periods of drought are a major environmental factor limiting successful field pea N symbiosis in Australia (GRDC, 2017). It was hypothesized that rhizobial strains would compete with each other for infecting host nodules and carbon resources for their growth

and survival under nutrient limited conditions. Further, it was suggested that rhizobial strains could gain potential benefits from these NRE communities infecting along with NRE under such environmental stress conditions. It was also found that a decline of rhizobia in large numbers in most of the drought affected legume fields causing poor nodulation and N fixation (Slattery et al., 2001). My work in chapter 4 further provided evidence for the significant reduction in N-fixation and total plant N in field pea plants. The same factors that reduce rhizobial colonisation (e.g. drought) may also reduce colonisation by NREs.

Here I hypothesized that the diversity of NRE would be higher in plants inoculated with only a single rhizobial strain since there would be less competition (more available niche space) compared to the multi-strain inoculated treatments (less available niche space) (Figure 5.1). Drought stress is expected to further reduce the diversity of NRE, with those found in nodules under water-stressed conditions hypothesised to exhibit drought-resistance (Vurukonda et al., 2016). Therefore, I expected the composition of NRE communities to vary, with some operational taxonomic units (OTUs) observed only under well-watered or water stressed conditions. To test these hypotheses, I performed bacterial 16S rRNA gene-based community profiling analyses of field pea root nodules treated with three rhizobial inoculations (WSM1455, competitors and mixed inoculation) and watering conditions (well-watered and reduced watering treatments) to observe variation in NRE diversity and community composition.

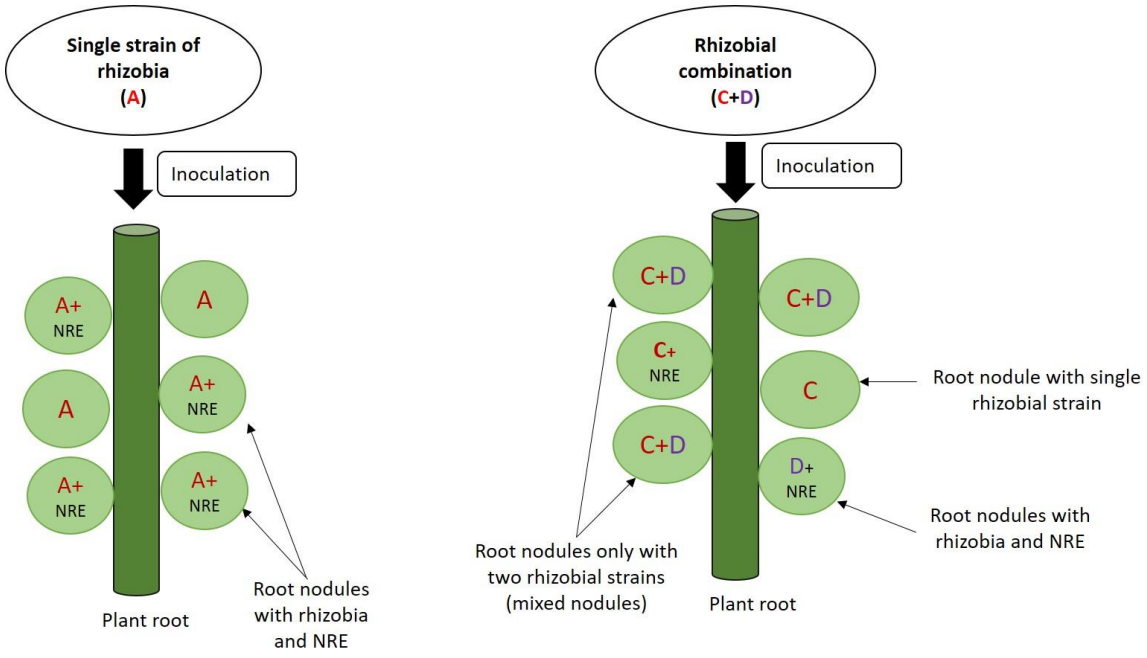

Figure 5. 1: Hypothetical diagram of predicted scenarios for niche space availability for NRE when inoculating the legumes with a single strain of rhizobia and rhizobial strain combination.

5.2 Materials and methods

5.2.1 Rhizobial strains, plant and soil material

The commercial inoculant *Rhizobium leguminosarum* WSM1455 (Group F) (GRDC, 2013) in peat-based formulation (NoduleN™ Peat - New Edge Microbials, Albury NSW, Australia) was used in the study. *Rhizobium leguminosarum* strains of RRI546 and RRI970 were selected as competitors from the natural rhizobial collection obtained from Department of Primary Industries, Victoria. Mixed cultures were prepared with mixing WSM1455 and both of competitors before inoculations. The field pea cultivar 'Twilight' (Hart Bros Seeds Pty Ltd., Junee Reefs NSW, Australia) was used in this chapter. The soil used in the experiment was described as Yarramundi Loam soil having reddish brown colour with light medium clay texture with a $pH_{(water)}$ of 6.0 (Isbell, 2016). More details on plant and soil properties and seed sterilization techniques can be found in chapter 4 (Section 4.2.1 Materials and methods).

5.2.2 Seed inoculation, planting and watering treatments

The commercial inoculant in peat form was prepared using cool water refrigerated at 4°C according to NoduleN™ Peat Instruction guide. Water used in the preparation of inocula was autoclaved at 120°C prior to cooling. The competitor strains were prepared in Yeast Mannitol Broth (YMB) (Somasegaran and Hoben, 1985), concentrations were measured to make equal cell numbers per ml (10^7/ml). The surface sterilized seeds were inoculated with single (WSM1455 and competitors separately) and mix (WSM1455 and both competitors together) of rhizobial strains. Plants were grown under a well-watered (80% field water capacity) and two cycles of reduced watering (60% FWC) conditions separately throughout the growing period of 8 weeks. The experiment layout was described in Table 4.2- chapter 4).

5.2.3 Plant harvest, collection of nodules and extracting bacterial DNA from root nodules

Eight-week-old plants were harvested. DNA was extracted from individual root nodules using Isolate II Plant DNA kit BIO 52070 (Bioline Pty Ltd, Australia). DNA concentrations were measured using NanoDrop 2000 Micro-volume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Delaware USA) by using at the absorbance at 260 nm. The extracted DNA concentrations were standardised as described in section 5.2.4.

5.2.4 Preparation of root nodule DNA for short read high-throughput sequencing

Table 5.1. shows the number of experimental units chosen from each treatment. Five nodule DNA extracts per each plant were pooled to make a composite DNA extract representing the population of nodules from an individual plant.

Table 5. 1: Selection and preparation of pooled DNA samples for Illumina Miseq (amplicon sequencing) analyses

Rhizobial Inoculation type	Watering Treatment	No of individual plants per treatment	~No of single nodule DNA extracts per plant	Final DNA concentration (ng/µl)	Final Volume of the sample (µl)
WSM1455	well-watered	7	5	5	15
WSM1455	reduced	5	5	5	15
competitors	well-watered	7	5	5	15
competitors	reduced	7	5	5	15
Mixed	well-watered	7	5	5	15
Mixed	reduced	5	5	5	15

Note: WSM1455= commercial inoculant *Rhizobium leguminosarum*, Competitors= *R. leguminosarum* strains (RRI546 and RRI970), Mixed= mixed inoculum with WSM1455 and competitors, Well-watered= 80% FWC, Reduced watering= 60% FW

DNA quantification was done using Qubit® 2.0 Fluorometer (Invitrogen^{TM} by Life Technologies, Carlsbad CA). Preparation of DNA samples for Qubit® analysis is described in detail with quantities in Appendix (4). The DNA samples were tested for PCR amplification of 16S rDNA using 27F and 1492R primers (Lane, 1991). The standardized DNA samples were arranged in 96-well PCR plates sealed with Microseal® 'B' films (BIO-RAD Laboratories, Australia). DNA samples were submitted to the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, NSW, Australia). Amplicons of the V4 region of the bacterial *rRNA* gene were generated using 515f (5'-GTGCCAGCMGCCGCGGTAA-3') and 806r (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011), purified using the Agencourt AMpure XP system (Beckman Coulter, Lane Cove, NSW, Australia) and genomic libraries were prepared using the Nextera XT Index Kit (Illumina, San Diego, CA, USA). Paired-end (2 x 251 bases) sequencing was performed on the Illumina MiSeq platform.

5.2.5 Bioinformatic processing of DNA sequences

To process the DNA sequencing data, we used the approach described by Bissett et al. (2016) with a few modifications. Contigs were generated from paired-end reads using the 'make.contigs' command in *mothur* (version 1.39.5) (Schloss et al., 2009). Initial quality

filtering removed DNA sequences containing ambiguous bases and/or homopolymers greater than eight bases in length. De novo operational taxonomic units (OTUs) at 97% sequence similarity were initially picked using numerically dominant sequences (observed at least four times) using the '-cluster_otus' command in *USEARCH* (version v8.1.1803) (Edgar, 2013). All quality-filtered sequences were mapped at 97% sequence similarity against representative sequences of these OTUs using the '-usearch_global' command in *VSEARCH* (version v2.3.4) (Rognes et al., 2016). Non-mapped sequences were subjected to a second round of de novo OTU picking, as above but only using sequences observed at least two times. All initially non-mapped sequences were then mapped against these newly picked OTUs, as above. Non-mapped sequences at this step represent singleton OTUs and were excluded from further analysis. Putative taxonomic identities for bacterial OTUs were generated using BLAST (v.2.6.0, Altschul et al. (1990)) to compare representative sequences for each OTU to a reference database of bacterial 16S rRNA gene sequences and taxonomic annotations (greengenes version 13_8, DeSantis et al. (2006)). OTUs matching mitochondrial or chloroplast DNA were removed prior to analysis, as were those OTUs assigned to the genus 'Rhizobium'. All remaining OTUs were considered to be NREs.

5.2.6 Statistical analyses

All analyses were performed using R version 3.6.1 (R Core Development Team). The analyses were performed using nodule DNA extracts of 38 individual field pea plants. For bacterial community analyses, sample-effort curves (supplementary Figure S5-1 in Appendix 4) were plotted using 'rarecurve' function in package 'vegan' (Oksanen et al., 2013). Random resampling of reads to normalize sampling depth per sample was not performed. Although the lack of normalization could impact the detection of effects (due to varying sequence depths of samples), Weiss et al. (2015) explained that the simulations are still valid for communities with low sequencing depths.

Indicator species analysis was performed to identify significant indicator OTUs (OTUs characteristic to particular inoculation type or watering condition) using 'indval' and 'multipatt' functions (package 'labdsv', (Roberts, 2007)). Indicator values (IV) quantify the exclusiveness of OTUs to a group, with '1' indicating complete exclusivity and '0' indicating no exclusivity. I identified indicator OTUs related to well-watered and reduced watering treatments and three different rhizobial inoculation types (WSM1455, competitors and mixed inoculation). Dufrêne and Legendre (1997) explained that the indicator OTUs with $P<0.05$ and IV>0.3 were

considered to be better indicators of the relevant environment. The variation in community composition was assessed with Principal Coordinates Analysis (PCoA) using 'wcmdscale' function ('vegan'). The effects of rhizobial inoculation type, watering treatment and their interaction were estimated on variation of non-rhizobial community composition based on Jaccard dissimilarities using permutational multivariate analysis of variance (PerMANOVA) with the 'adonis' function ('vegan'). The function 'adonis' was used on three subsets of rhizobial inoculation types to perform pairwise comparisons of NRE community composition in two watering treatments. Using the nonrhizobial endophyte matrix, three types of diversity indices were estimated; 1. Expected non-rhizobial richness for each sample using Chao.1 index (Chao et al., 2005) with 'estimateR' function (package 'vegan'). 2. The species evenness and richness of non-rhizobia using the function 'diversity' (Shannon species diversity, Shannon (1948)) and 3. the Pielou evenness (Pielou, 1966) was calculated using the function 'specnumber' and shannon diversity (see below equation);

Species Evenness= Species diversity/ log (Species richness)

The effects of rhizobial inoculation type, watering condition and their interactions on NRE diversity and evenness were estimated using linear models ('Two-way ANOVA'). Multiple comparisons were made using the function 'emmeans' ('emmeans' package, Lenth aet al. (2019)) to determine how non-rhizobial bacterial richness, diversity and evenness differed among rhizobial inoculation types and watering treatments. All the figures were constructed using the package 'ggplot2' (R 3.6.1) (Wickham, 2009).

5.3 Results

5.3.1 The diversity of non-rhizobial endophytes (NRE) increases in mixed rhizobial treatment (WSM1455+competitors) under reduced watering conditions

A total of 1,987,473 forward reads paired with reverse reads were obtained before any quality control. Initial quality filtering and removal of putative chimeras and singletons removed 108,312 reads. Following these steps, a total of 1,879,161 reads remained. After removing rhizobial, mitochondrial and chloroplast OTUs, there were 40,246 reads for non-rhizobial endophytes which were grouped according to the inoculation and watering treatment as in Table 5.2.

Table 5. 2: Number of non-rhizobial reads categorized under each inoculation type and watering conditions (well-watered at 80% FWC and water stress at 60% FWC)

Inoculation Treatment	Watering conditions	No of non-rhizobial reads
WSM1455	Well-watered	5255
WSM1455	Reduced	6868
competitors	Well-watered	7077
competitors	Reduced	9786
Mixed	Well-watered	7840
Mixed	Reduced	3420

A total of 1193 bacterial OTUs were observed across 38 plants. No evidence obtained for the type of rhizobial inoculation and watering conditions had significant effects on Shannon diversity of NRE (P=0.09 and P=0.89 respectively, Two-way ANOVA, Table 5.3).

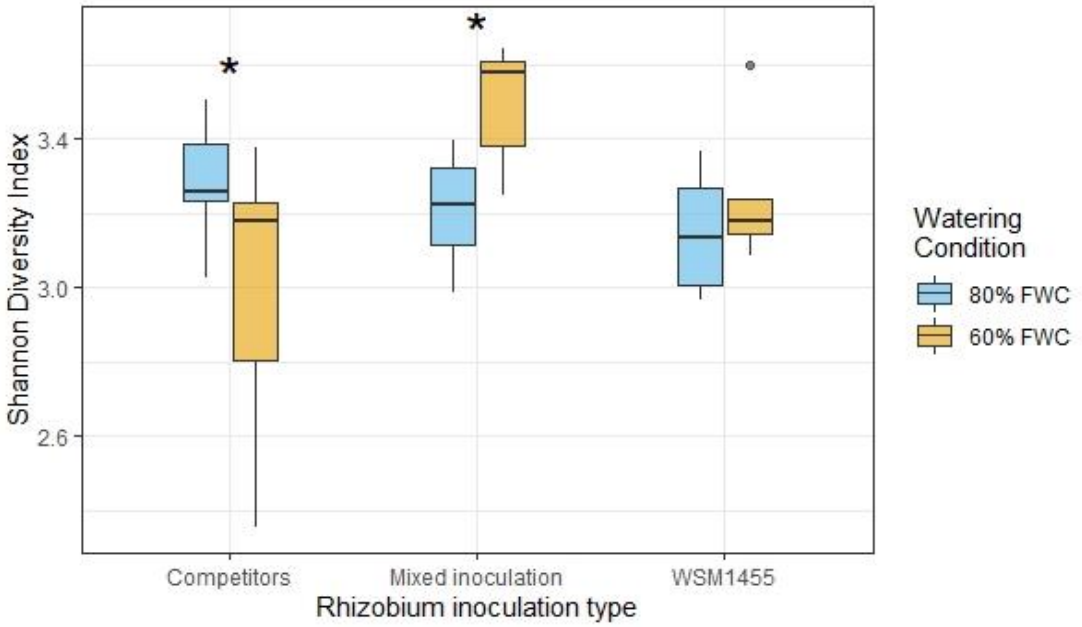

Figure 5. 2: Box plots showing the estimated (Shannon) diversity of non-rhizobial nodule endophytic bacterial communities in three rhizobial inoculation types (WSM1455=Commercial inoculant, Competitors= *R. leguminosarum* strains of RRI546 and RRI970 & Mixed inoculation= mix of the WSM1455 and competitors) (n=7 each) under well- watered (80% FWC) and reduced watering (60%FWC) conditions. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or when dots are present, extreme values within 1.5 times the interquartile range. $P_{inoculation\ type}$= 0.09, $P_{watering}$= 0.89, $P_{ino\ x\ watering}$ = 0.005 (Two-way ANOVA). Asterisks (*) are indicated on plots where comparisons between pairs are significant.

However, there was a significant interaction between the rhizobial inoculation type and watering treatment (P<0.01, Two-way ANOVA, Table 5.3, Figure 5.2). Under well-watered conditions (80% FWC), there was not a significant variation in NRE diversity among inoculation types whereas in the reduced watering conditions (60% FWC), the diversity of NREs significantly varied between mixed inoculation treatment and competitor treatment (~9.4% increase in Mixed inoculation, P<0.01, Multiple comparisons 'emmeans', [Mixed v. competitors]). Moreover, the comparisons between mixed inoculation in well-watered and water stressed conditions revealed a significant increase (12.5%) in NRE diversity under reduced watering status (P=0.03, [Mixed $_{80\%FWC}$ v. Mixed $_{60\%FWC}$], Multiple comparisons). There was also approximately 3% reduction in NRE diversity obtained in Competitors $_{60\%FWC}$ treated plants over Competitors $_{80\%FWC}$ (P=0.02, Multiple comparisons). A significant difference of NRE diversity between single WSM1455 infected plants under two watering treatments was not observed. (P=0.42, [WSM1455$_{80\%FWC}$ v. WSM1455$_{60\%FWC}$], Multiple comparisons with 'emmeans').

5.3.2 NRE richness is marginally altered by watering condition but not by rhizobial inoculation type

Non-rhizobial bacterial richness was not affected by the type of rhizobial inoculation or the interaction between inoculation type and watering treatment (P>0.05, Two-way ANOVA, Table 5.3, Figure 5.3). Further, there was a marginally non-significant effect observed for watering condition on the NRE richness (P=0.06, Two-way ANOVA, Table 5.3, Figure 5.3).

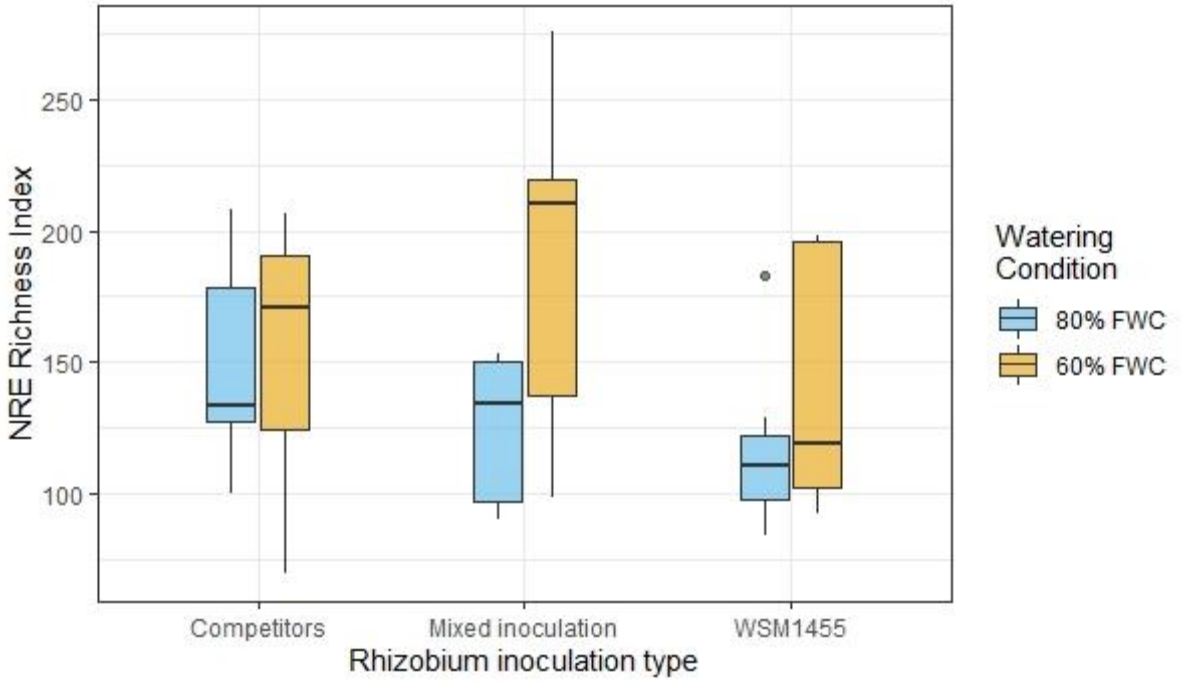

Figure 5. 3: Box plots showing the estimated richness of non-rhizobial nodule endophytic bacterial communities in three rhizobial inoculation types (WSM1455 = Commercial inoculant, Competitors = *R. leguminosarum* strains of RRI546 and RRI970 & Mixed inoculation = mix of the WSM1455 and competitors) (n=7 each) under well-watered (80% FWC) and reduced watering (60%FWC) conditions. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or when dots are present, extreme values within 1.5 times the interquartile range. $P_{inoculation\ type}$= 0.31, $P_{watering}$= 0.06, $P_{ino\ x\ watering}$ = 0.25 (Two-way ANOVA).

Table 5. 3: Summary of analysis of variance (Two-way ANOVA) showing the effects of rhizobial inoculation type and type of watering condition on OTU diversity, richness and evenness of non-rhizobial nodule endophytic bacterial communities

Source of variation	Species Diversity			Species Richness			Species Evenness		
	Df	**F**	**P**	**DF**	**F**	**P**	**DF**	**F**	**P**
Rhizobial Inoculation Type	2,32	2.51	0.09†	2	1.2	0.31	2	3.82	0.03*****
Watering	1,32	0.02	0.89	1	3.78	0.06†	1	0.30	0.58
Inoc X watering	2,32	6.04	<0.01 ******	2	1.42	0.25	2	4.03	0.03*

DF= degrees of freedom, ******=*P*<0.01, *****= *P*<0.05 and † = *P*<0.1

5.3.3 Evenness of NRE was significantly affected by rhizobial inoculation type and interaction between inoculation type and watering condition

There was a significant interaction of rhizobial inoculation type and watering condition affecting the NRE evenness (P=0.03, Two-way ANOVA, Table 5.3). Further, the evenness of NRE OTUs was higher in mixed inoculation treatment compared to competitor strain treatment under water stressed conditions (P<0.01, Multiple comparisons, Figure 5.4). Under well-watered conditions, NRE OTU evenness did not differ significantly among rhizobial inoculation types (P>0.05, Multiple comparisons).

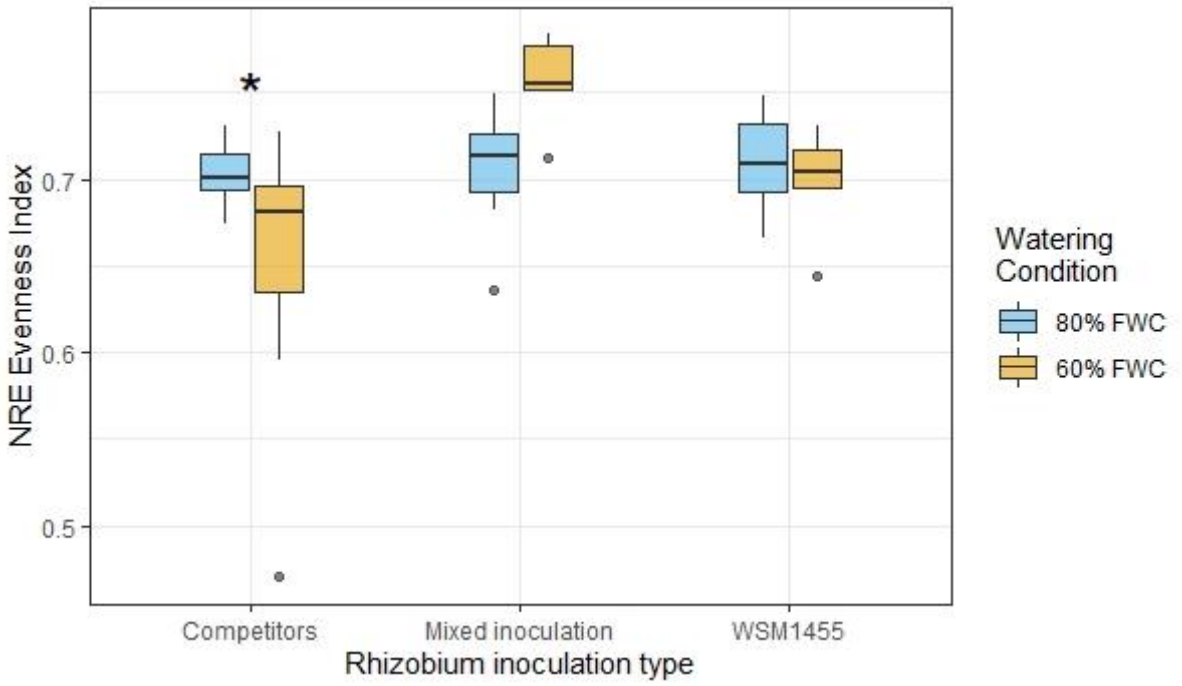

Figure 5. 4: Evenness index of non-rhizobial nodule endophytic bacterial communities in three rhizobial inoculation types (WSM1455 = Commercial inoculant, Competitors = *R. leguminosarum* strains of RRI546 and RRI970 & Mixed inoculation = mix of the WSM1455 and competitors) (n=7 each) under well- watered (80% FWC) and reduced watering (60%FWC) conditions. Asterisks (*) are indicated on plots where comparisons between pairs are significant. The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range. When dots are present, extreme values within 1.5 times the interquartile range. $P_{\text{inoculation type}}$= 0.03, P_{watering}= 0.58, $P_{\text{ino x watering}}$ = 0.03 (Two-way ANOVA).

There was approximately 4% reduction in OTU evenness of NRE in competitors infected plants under reduced watering conditions (P=0.03, Multiple comparisons, [Competitors $_{80\%FWC}$ v. Competitors $_{60\%FWC}$]). In contrast, there was no significant increase of NRE evenness in mixed inoculated plants under reduced watering conditions compared to well-watered conditions (P=0.07, Multiple comparisons, [Mixed $_{80\%FWC}$ v. Mixed $_{60\%FWC}$]).

5.3.4 Identification of indicator OTUs of NRE associated with watering treatments and rhizobial inoculation treatments

Specific indicator OTUs of NREs associated with either well-watered and water-stressed conditions were identified. There were 19 significant OTUs, of which 8 aligned to the well-watered treatment and 11 were associated with reduced watering treatment. The OTU indicator values were >0.48 across both watering treatments and there was a significant variation in families of NRE occurring in each watering condition (Table 5.4).

Table 5. 4: OTUs of NRE significantly associated with two watering treatments (well-watered -80%FWC and reduced watering- 60% FWC). The table includes type of watering treatment, the name of the OTU, indicator value (IV) range from 0 to 1 where highest values are associated with stronger indicators of the particular watering condition, *P* value indicating the significance of occurrence of particular indicator OTU, the taxonomy of the OTU (based on the sequence comparison using BLAST), query cover with percentage of sequence cover, percentage ID (aligned residues). The Subject Sequence ID (SseqID) is the accession of the database sequence providing the best match.

				Best matched taxa using BLAST			
Watering Treatment	**OTU ID**	**IV**	***P***	**Taxonomy**	**Query cover**	**ID%**	**sseqID**
Well-watered	16Sall_OTUa_113	0.716	0.015	Oxalobacteraceae	100	100	4299496
	16Sall_OTUb_27164	0.636	0.03	*Rhodanobacter lindaniclasticus*	100	93	805823
	16Sall_OTUb_3030	0.627	0.049	*Acinetobacter johnsonii*	100	100	4481710
	16Sall_OTUb_5216	0.617	0.013	*Mucilaginibacter gracilis*	100	98	4397087
	16Sall_OTUb_4791	0.577	0.01	*Phenylobacterium* sp.	100	97	2066504
	16Sall_OTUb_21732	0.555	0.032	*Rhodanobacter terrae*	100	94	4482513
	16Sall_OTUb_3406	0.535	0.028	Acidobacteriaceae	100	99	4413492
	16Sall_OTUb_43909	0.488	0.044	*Pedobacter* sp.	99.6	99	790094
Reduced watering	16Sall_OTUa_159	0.782	0.002	*Kribbella* sp.	100	100	4378288
	16Sall_OTUb_53668	0.737	0.007	*Terracoccus* sp.	100	97	4468156
	16Sall_OTUa_4954	0.673	0.009	*Bacillus firmus*	100	99	4434972
	16Sall_OTUa_202	0.642	0.005	*Flavisolibacter* sp.	100	100	4459870
	16Sall_OTUa_5141	0.629	0.03	*Micromonospora coxensis*	100	98	804849
	16Sall_OTUd_4019	0.618	0.017	*Kaistobacter* sp.	100	97	4302797
	16Sall_OTUb_33421	0.567	0.03	*Mucilaginibacter jinjuensis*	100	97	4427760
	16Sall_OTUb_31053	0.523	0.039	Betaproteobacteria (Ellin6067)	100	99	4309772
	16Sall_OTUa_2333	0.485	0.032	*Sphingomonas* sp.	100	99	1133675
	16Sall_OTUa_2569	0.485	0.03	*Flavisolibacter* sp.	100	99	849080
	16Sall_OTUa_5010	0.485	0.036	Isosphaeraceae	100	100	1049509

Table 5. 5: NRE OTUs significantly associated only with WSM1455 or Mixed inoculation and in the absence of the commercial inoculant (Competitors only). The table includes type of rhizobial inoculation, the name of the OTU, indicator value (IV) range from 0 to 1 where highest values are associated with stronger NRE indicators of the particular rhizobial inoculation, *P* value indicating the significance of occurrence of particular indicator OTU, the taxonomy of the OTU (based on the sequence comparison using BLAST), query cover with percentage of sequence cover, percentage ID (aligned residues). The Subject Sequence ID (SseqID) is for accessing the best matched sequence database.

Type of rhizobial inoculation	OTU ID	IV	*P*	Best matched taxa using BLAST Taxanomy	Query cover	ID%	sseqID
Competitors	16Sall_OTUa_63	0.706	0.001	*Kaistobacter* sp.	100	99.6	1702117
	16Sall_OTUb_5216	0.693	0.001	*Mucilaginibacter gracilis*	100	98	4397087
	16Sall_OTUb_4489	0.689	0.039	*Janthinobacterium lividum*	100	98	1037037
	16Sall_OTUb_43909	0.598	0.002	*Pedobacter* sp.	99.6	99	790094
	16Sall_OTUa_2513	0.587	0.008	*Azospirillum*	100	100	21846
	16Sall_OTUd_9926	0.586	0.003	*Pedobacter cryoconitis*	100	96	4397089
	16Sall_OTUa_1368	0.547	0.04	*Dongia mobilis*	100	96	800317
	16Sall_OTUb_5485	0.535	0.012	*Kaistobacte*r sp.	100	97	4452571
	16Sall_OTUb_54682	0.463	0.041	Solibacteraceae	96	99	534914
	16Sall_OTUe_91	0.463	0.047	*Dyella marensis*	98	98	2132253
WSM1455 only and Mixed inoculation	16Sall_OTUd_3401	0.701	0.029	*Sphingomonas wittichii*	100	97	238508

To identify specific OTUs associated with either the presence or absence of the commercial inoculant, indicator OTUs in WSM1455 only and mixed inoculation treatments have been analyzed (see Table 5.5). The higher OTU indicator values suggested highly associated species in each of the inoculation treatments. Of the 11 indicator OTUs recorded, only one indicator OTU (*Sphingomonas wittichii*) was specifically associated (indicator value of 0.7) with commercial inoculant added treatments.

The effects of watering treatment and the rhizobial inoculation type on NRE community composition were evaluated using Jaccard dissimilarities (PerMANOVA). There were significant effects of both watering treatment (PerMANOVA, $F_{1,37}$= 1.5, R^2=0.04, P=0.001) and the type of rhizobial inoculation (PerMANOVA, $F_{2,37}$= 1.13 R^2=0.06, P=0.042). There was also a significant interaction between watering treatment and rhizobial inoculation type on NRE community composition (PerMANOVA, $F_{2,37}$= 1.33 R^2=0.07, P=0.002).

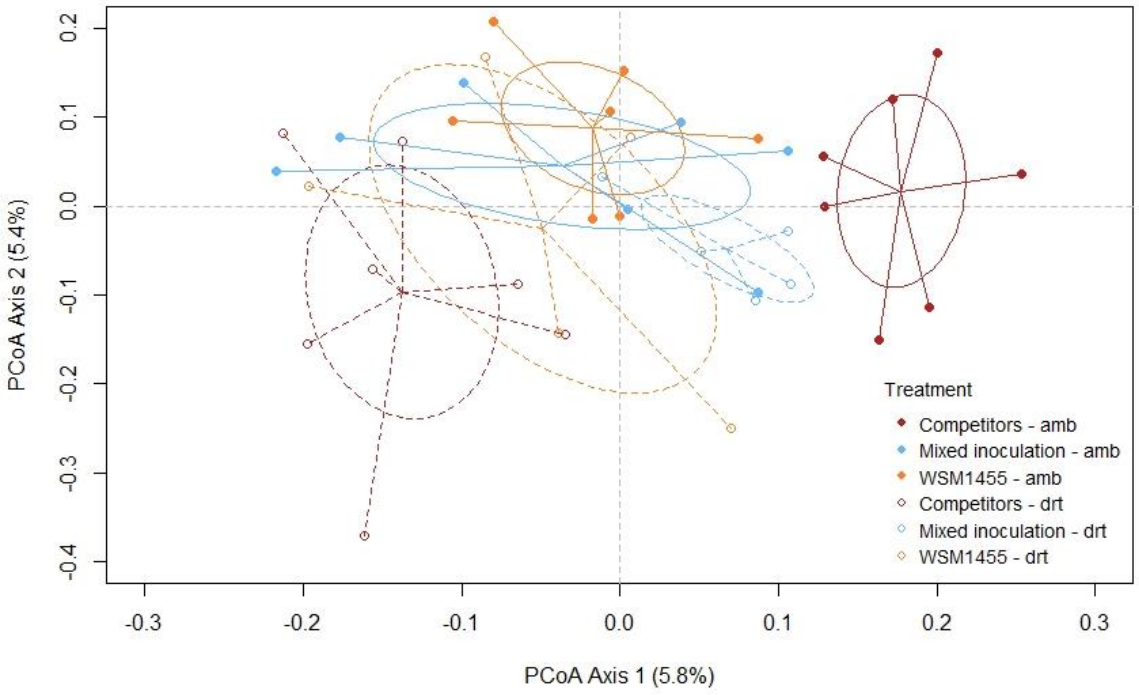

Figure 5. 5: PCoA (MDS) plot of non-rhizobial nodule endophyte (NRE) communities (bacteria) by 'Treatment' with rhizobial inoculation type (WSM1455 = Commercial inoculant of *Rhizobium leguminosarum*, Competitors = *R. leguminosarum* strains of RRI546 and RRI970 & Mixed inoculation = mix of WSM1455 and Competitors) and well-watered treatment at 80%FWC (amb) and reduced watering at 60%FWC (drt)) based on Jaccard distances. Each point represents a NRE community pooled at the level of an individual pea plant. Standard error ellipses show 95% confidence areas where each point is connected to the group centroid. The proportion of variation is explained by each plot axis in percentage values.

Under both well-watered and reduced watering conditions, the inoculation with competitor strains resulted in well-separated distinctive NRE communities (Figure 5.5, $F_{2,11}$= 1.7, R^2=0.12, P=0.002, [Competitors $_{80\%FWC}$ v. Competitors $_{60\%FWC}$], pairwise comparisons by inoculation). There were distinct NRE communities when inoculated as mixed inoculum under two watering conditions (Figure 5.5, $F_{2,11}$= 1.3, R^2=0.12, P=0.002, [Mixed $_{80\%FWC}$ v. Mixed $_{60\%FWC}$]). However, I did not observe significantly different NRE communities for the treatment with WSM1455 under well-watered and water-stressed conditions (Figure 5.5, $F_{2,11}$= 1.04, R^2=0.09, P=0.32, [WSM1455 $_{80\%FWC}$ v. WSM1455 $_{60\%FWC}$]).

5.4 Discussion

5.4.1 NRE diversity significantly increased in mixed rhizobial inoculations in field pea hosts under water stress

The diversity of NRE was greater in mixed rhizobial inoculation (WSM1455+Competitors) treatments whereas it significantly decreased in inoculation only with competitors under water-stressed conditions. This observation was in contrast to one of the proposed hypotheses that competitors would leave more available niche space for NRE in nodules than mixed inoculations. The occurrence of WSM1455 was more frequent in nodules of the plants with mixed inoculations (chapter 4). Therefore, it might be the fact that WSM1455 would not impose strong competitive pressure on NRE under drought which allow more NRE to colonize in nodules. The potential reasons are not yet explored and could be further studied in future to determine whether WSM1455 has any influence on NRE colonization and could drought affect these interactions. However, it was also observed that under well-watered conditions, neither the Shannon diversity nor richness of NRE significantly varied among different rhizobial inoculation types. Having diverse NRE community in mixed rhizobial inoculation under water stress might protect plants from the negative impacts of stressed conditions by producing different anti-pathogenic substances (Lau and Lennon, 2011). It has been found that the host plants might benefit from nodule endophytes either via biosynthesis of anti-stress biochemical compounds (Schulz, 2006) or activating or upregulating host stress resistance genes (Bailey et al., 2006). Therefore, having diverse NREs in mixed rhizobial infection treatment under water stress might benefit a plant via alleviating adverse effects of drought (Lata et al., 2018). It could also be possible that colonization of root nodules by NRE might be independent from plant selection mechanisms and they just enter the root nodules with rhizobial symbionts. Decrease in NRE diversity in competitor strain treatment in my experiment could be due to the intense

inter-strain competition under water-stressed conditions (Kiers et al., 2003) opening up less window for NRE to colonize in available nodule spaces. The effect of watering condition was marginally non-significant on NRE richness. Although the actual mechanism for this observation was not explored, one possibility could be that the drought tolerant NRE colonize host nodules in large numbers under water limited conditions (Naylor and Coleman-Derr, 2018). NRE richness increased in mixed rhizobial treatment under reduced watering conditions by 70% compared with mixed inoculations under well-watered conditions. However, it is still unclear whether this higher NRE colonization would benefit the host plants. Observing less variation of NRE richness in mono-inoculation treatments of WSM1455 could possibly be due to the pea hosts benefitting more from the commercial inoculant as an effective symbiont (as per Chapter 4 results). The host plants would not be in need for increasing the interactions of other endophytic communities specially during environmental stress conditions such as water stress (Vuong et al., 2017) when they already interact with an effective symbiont.

It was also observed that the NRE species evenness was affected by the type of rhizobial inoculation where the evenness in mixed rhizobial treatment significantly increased compared to competitor treatment under water stressed conditions. In line with my findings, Peñuelas et al. (2012) found that the evenness of endophytic bacterial community increased in the leaves of *Quercus ilex*, the dominant tree species of Mediterranean forests. One suggested possibility was that drought has caused the loss of dominance of some species. Further, my work showed that the interaction of watering condition and inoculation type was a significant predictor of NRE evenness in field pea hosts. My observations were also in line with another study by Gadhave et al. (2018) where they found that the seed of Green sprouting broccoli inoculated with plant growth promoting rhizobacterium, *Bacillus amyloliquefaciens* increased the diversity and evenness of endophytic bacterial communities. Although my data do not allow to characterize the pathway by which the effect of the water limited conditions altered richness, nor the NRE diversity or evenness, these findings offer a promising line for future investigation.

5.4.2 Indicator species of NRE varied among watering treatments and rhizobial inoculation types

The most strongly associated NRE OTUs in well-watered treatment (having the indicator value of 0.7) belonged to the family Oxalobacteraceae (order Burkholderiales) which is one of the dominant root-nodule endophytic bacterial families in legumes (Sawada et al., 2003). The genus *Burkholderia* is studied for its ability of promoting growth and N-fixation in legume hosts

(Zgadzaj et al., 2016). Similarly, the non-rhizobial endophyte OTUs belong to families Pseudomonaceae, Sphingobacteriaceae and Acidobacteriaceae which could also contribute as PGPR (plant growth promoting rhizobacteria) in N-fixing legumes in general (Santoyo et al., 2016). Under reduced watering conditions, the most strongly associated OTU is identified as *Kribbella* sp. which has been observed as a drought tolerant endophytic bacteria regulating plant metabolism (Hamedi and Mohammadipanah, 2015). Further, several other significant indicator OTUs associated with reduced watering treatment belong to family Sphingobacteriaceae (eg. *Kaistobacter* sp., *Sphingomonas* sp., *Flavisolibacter* sp. and *Mucilaginibacter* sp.). This root endophytic family is well-known for protecting the plants against bacterial wilt, especially *Kaistobacter* sp. is found to act as a disease suppressing endophyte against soil-borne pathogens such as *Rhizoctonia solani* and *Fusarium oxysporum* (Liu et al., 2016). *Bacillus firmus,* significantly associated in root nodules of our reduced watering treatments, was observed in other studies to alleviate salt-stress, enhance biomass yield, flavonoid contents and increase nutrient uptake in soybean (*Glycine max* L.) (El-Esawi et al., 2018). Therefore, most of the significant NRE OTUs associated with reduced watering treatment, may have roles of stress tolerance, acting against pathogens, promoting plant growth and nutrient uptake (Rubin et al., 2017). However, it is suggested that the isolation of these particular NREs and re-inoculating into the pea hosts in future work would reveal the actual roles of these endophytes in plant growth and function.

The specific indicator NRE OTUs associated with different rhizobial inoculation types, indicated 10 significant OTUs that were associated with competitor rhizobia including *Kaistobacter* sp., *Pedobacter* sp., and *Mucilaginibacter gracilis.* I could also observe an association of N-fixing *Azospirillum* sp. in competitor strain inoculations. Though the actual mechanism of *Azospirillum* sp. is not explored in my study, other studies have demonstrated a synergistic interaction with *Rhizobium* sp. in root nodules enhancing N-fixation by formation of epidermal cells that differentiate into root hairs to acquire more N-fixing rhizobia (Steenhoudt and Vanderleyden, 2000, Yahalom et al., 1987). Further, I found only one significant indicator OTU of NRE associated with commercial rhizobium inoculant treatment, identified as *Sphingomonas wittichii*. Although the previous studies found that *Sphingomonas* sp. is capable of producing gibberellins and indole acetic acid to promote plant growth (Khan et al., 2014), the role of this endophytic bacterium with commercial rhizobial inoculant in this case is still unexplored. Alternatively, observing only one indicator NRE OTU (belonging to *Sphingomonas* sp.) in WSM1455 associated treatments and more NRE OTUs with competitor

rhizobial treatment might be that *Sphingomonas* sp. could dominate over the other NRE in nodules by producing antibiotics (Innerebner et al., 2011, White et al., 1996). There is also a possibility that specific OTUs of NRE associated with WSM1455 and/or competitor strains might have been introduced at the time of inoculation.

5.4.3 The type of rhizobial inoculation and reduced watering treatment significantly influenced the composition of non-rhizobial endophytic bacterial communities

The NRE community composition in field pea nodules was affected by the type of rhizobial treatment and reduced water. There was a significant interaction between the rhizobial inoculation type and watering condition affecting NRE composition. My results showed distinctive NRE communities associated with competitor rhizobial treatment under well-watered condition compared to those in water-stressed conditions. In contrast, I did not observe significant separation of NRE communities associated with commercial rhizobial inoculation treatment under both the watering conditions. The reason of having distinct NRE communities with competitor rhizobial treatment under different watering conditions is unknown. Further my work showed a greater number of indicator NRE OTUs associated with competitor treatment than WSM1455 treatment. One possibility is that more NREs are successful colonizing under competitor treatment than WSM1455 treatment but the actual mechanism for this observation is not yet explored. Despite the dominance of commercial inoculant in mixed inoculation treatment, I could observe distinct NRE communities in well-watered conditions compared to those present in water-stressed conditions. More research is needed to disclose the actual mechanisms for this observation. Nonetheless, the variation in symbiotic (N-fixing) efficiencies, stressful environmental conditions and variation in host plant preferences could collectively manipulate the NRE community composition in cropping fields (Barrett et al., 2015).

5.4.4 Key findings and proposed future work

In conclusion, my work suggests that NRE bacterial communities associated in field pea root nodules are likely to vary among different rhizobial inoculation types and soil water status. I observed that the NRE diversity in mixed inocula (WSM1455+Competitors) can be affected by the interactive effect of rhizobial strains and watering condition while no evidence that the NRE diversity or community composition varied in single inoculations of commercial inoculant in field pea plants either well-watered or reduced watering treatments. Moreover, the water stress and rhizobial inoculation type appeared to be affecting NRE diversity in different aspects, such

as NRE richness was altered by water stress and NRE evenness was affected by the rhizobial inoculation type. The current study does not address the question of whether the elevated water stress conditions facilitate greater NRE diversity regardless of rhizobial colonization in pea root nodules. Since the NRE communities are both diverse in function and large in numbers in the rhizosphere, the potential functional roles of these communities need to be explored in future studies. These may determine whether some NREs protect legumes from pathogens under water stress. Neither the specific roles of NRE communities under water stress nor the mechanisms of the host plants to select beneficial NRE communities proposed in previous sections have been tested. Therefore, partner choice of the host plants could be explored by inoculating selected NRE species under varying environmental conditions (e.g. under drought) to see whether the host plant filter NRE according to the environment. It would also be important to look at whether there could be an interaction with the rhizobial inoculant under particular environmental constraints for a specific NRE community to be selected by the host plant. Further work should also look at how well a commercial rhizobial inoculant interacts with other NRE under field conditions and whether these synergistic (or antagonistic) interactions significantly affect overall legume growth and function. Having distinctive NRE communities in competitor strain treatment and mixed rhizobial treatment under different watering conditions provides insights into natural defense and growth promotion mechanisms associated with legume hosts under stressful environment gradients. Future studies focusing on synergistic interactions between beneficial NRE communities and effective N-fixing rhizobial inoculants would generate further understanding on the potential benefits of using the mixed (NRE and rhizobia) inocula in legume fields under stressful environmental conditions.

Chapter 6: General Discussion

6.1 Summary of the main research objectives and proposed questions

The main objectives of this project were to gain a better understanding of interactions among *Rhizobium leguminosarum* strains, observe interactions with other bacteria in nodules and to investigate whether these interactions could have significant effects on overall N nutrition and growth promotion of field pea. Further, this work aimed to determine whether environmental constraints (such as drought) could alter these rhizobial interactions.

The following specific questions were set out to address the aims of the study:

1. To what extent does the symbiotic interaction of *Rhizobium leguminosarum – Pisum sativum* allow nodulation by multiple rhizobial strains in a single root system?
2. To what extent do *R. leguminosarum* strains co-occupy single root nodules?
3. Does strain similarity, inferred from genetic similarity, affect the outcome of interactions within root systems and single nodules?
4. If multi-strain colonisations are successful, then will these multi-strain infections fix more N compared to single strain inoculations?
5. To what extent does a rhizobial inoculant for field pea retain its nodulation and N fixing efficiency in the presence of competitor strains?
6. Does drought alter the interactions between commercial and competitor strains of *R. leguminosarum* and impact overall N nutrition of field pea hosts?
7. To what extent do drought and interactions between rhizobial strains affect the diversity of non-rhizobial endophytes in pea root nodules?

6.2 Key findings

Overall the current work has demonstrated the importance of evaluating the inter-strain interactions of *R. leguminosarum* in field pea N nutrition. The results of this study showed that the rhizobial genetic similarity could be a useful tool for developing new, diverse inoculants, narrowing possible rhizobial combinations down to a reasonable number to inoculate together. When inoculated in pairs, distantly related strains of *R. leguminosarum* formed more nodules in pea plants compared to closely related pairs indicating better inter-strain associations in root colonisation (chapter 2). However, while rhizobial N fixation and plant biomass were greater when inoculated with pairs of isolates, as opposed to single isolates, the effect was not observed to differ depending on the genetic similarity of the rhizobial pairs. Further, enterobacterial

repetitive intergenic consensus (ERIC) PCR used in my work could be an effective molecular tool to identify the presence of *Rhizobium leguminosarum* strains (RRI strains) having unique banding patterns.

Infection of individual nodules by multiple rhizobial strains was infrequently observed (chapter 3). It could also be possible that some rhizobial strains were not detected in ERIC PCR because they might be quite infrequent relative to the most abundant strains that were detected. Fluorescent labelling of rhizobial plasmid proteins could be an alternative approach for identifying strains under fluorescence microscope (Duodu et al., 2008). Although, I hypothesized that inoculation of distantly related strains would frequently form mixed infections, rhizobial genetic similarity was not a significant predictor for the frequency of mixed nodule infections. However, inoculating distantly related strains showed higher nodulation in the same root system and increased N fixation compared to closely related strains.

I found no evidence to demonstrate that the rhizobial genetic similarity was a better predictor for synergistic interactions in enhancing plant biomass. However, I could identify some *R. leguminosarum* strains used in the study were not efficient N fixers either inoculated singly or in a pair (e.g. RRI1204). This observation was in line with some previous studies (such as (May and Bohlool, 1983, Somasegaran and Bohlool, 1990)) which demonstrated that the some competitive rhizobial isolates are efficient in nodulation but contribute less to N fixation. Moreover, my work demonstrated that the effective N fixing strains should also be successful among ineffective competitor strains in cropping soils to form nodules.

As a strategy for enhancing N fixation, inoculating legume crops with commercially developed rhizobial strains is a well-known agricultural practice in the world (Catroux et al., 2001). It has also been suggested that most of the ineffective rhizobia found in soils could impose intense competition on introduced strains (Denton et al., 2002). The observations of inter-strain interactions of rhizobia in chapters 2 and 3 led to extend my work looking at whether the commercial inoculant could be successful in colonising pea plants in the presence of competitor rhizobial strains. Since drought is a major environmental constraint in most of the Australian legume grown fields (GRDC, 2017, Williams et al., 2002), this study also assessed whether drought would alter these inter-strain interactions compared to well-watered conditions (chapter 4). In line with the proposed hypotheses for chapter 4, the commercial inoculant WSM1455 had less N benefits to the host plant in the presence of competitor rhizobial strains and drought. Further, cultivar Wharton had higher plant N and biomass than cultivar Twilight irrespective of

watering condition. Therefore, cultivar Wharton could be further evaluated for its capacity in producing higher grain yields in water-stressed cropping soils.

Current work has also examined the compositional shifts of non-rhizobial endophyte communities (NREs) in pea root nodules. Under drought conditions, NRE diversity increased in mixed rhizobial treatment (WSM1455+competitors) whereas it decreased in competitor strain treatment. It was suggested that the intense competition among competitor strains for nodulation (Kiers et al., 2003) might have prevented the NREs from entering to nodules. Further, I found that some NRE OTUs were strongly associated with particular rhizobial or watering condition. For example, *Kribella* sp. strongly associated with drought conditions whereas *Sphingomonas wittichii* significantly associated with commercial inoculant (WSM1455) treated plants. The results of the current study were not sufficient to demonstrate the specific roles of these NREs associated with particular rhizobial/watering treatments. More future experimental approaches to exploit beneficial NREs are described in detail in section 6.3.

6.3 Future directions

6.3.1 Investigating plant resource allocation on effective versus ineffective rhizobial symbionts in root nodules

The findings of the current study (variation of rhizobial strain effectiveness), together with quantifying plant resource allocation in nodules among different rhizobial strains, may provide for improved predictions of efficient N-fixing rhizobial strains. This approach can also be extended examining the interactions of these rhizobial strains among different pea genotypes. It will also help to select/ breed pea cultivars having the ability of exploiting more N benefits from rhizobia through efficient sanctioning mechanisms. Host resource allocation (carbon and oxygen) is important for growth and function of rhizobia in nodules (Denison and Kiers, 2004). Previous studies have shown that the oxygen supply to the nodules with no N fixation could be sanctioned by the host plant (Kiers et al., 2003). Chapters 2 and 3 of this study demonstrated the variation of N fixing efficiency of different *R. leguminosarum* strains infected in field pea nodules. The question raised is whether the field pea hosts would be able to identify the non-fixing *R. leguminosarum* strains over the better fixing strains. Secondly, if the pea hosts could identify the non-fixing strains would the plants sanction the nutrient supply for non-fixing nodules. Therefore, it is suggested to measure the amount of carbon (supply of photosynthate) and oxygen supply to nodules infected with particular strains. Therefore, I hypothesize that:

a) Inefficient rhizobial strains would have low amount of carbon for their growth and reproduction.

b) Plants would apply sanctions on less mutualistic rhizobia through limiting oxygen supply to nodules (Figure 6.1)

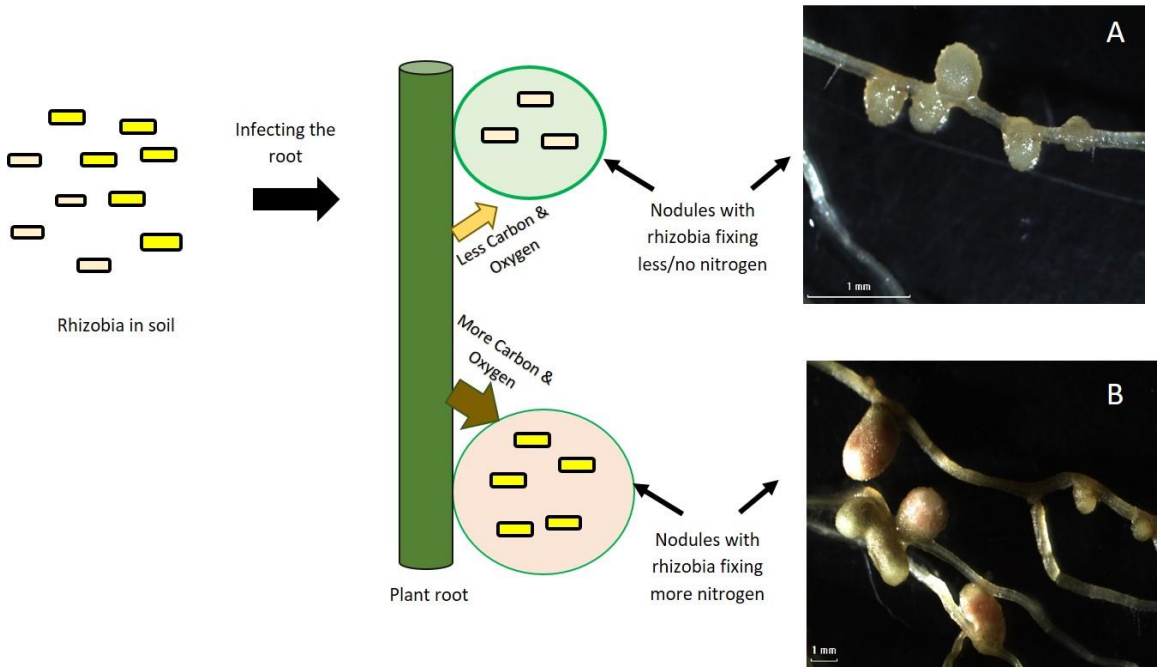

Figure 6. 1: Hypothetical diagram showing the carbon and oxygen supply among beneficial and less beneficial rhizobial strains. The image at top right corner (A) shows the pale green color nodules which had zero nitrogenase activity and image (B) shows the pink color nodules with high nitrogenase activity measured using acetylene reduction assay in chapter 3.

Future experiments could use $^{13}CO_2$ enrichment technique for analyzing ^{13}C discrimination in different root nodules (stable isotope techniques) (Subedi et al., 2006) to determine whether there could be variation in host carbon supply. To explore whether the host plant sanctions the respiratory oxygen to ineffective nodules (with no N fixation), the rate of oxygen supply could be measured using fiber optic oxygen sensors (Ott et al., 2005).

6.3.2 Development of plant cultivars which attract multiple N fixing rhizobial symbionts

Exploiting symbiotic genes of legumes which attract more rhizobial partners could be used to breed novel plant cultivars in the future. Current findings showed that mixed nodule infections were only associated with cultivar Wharton (chapter 4). It might be that the cultivar Wharton facilitate more rhizobial strains in nodules by providing more carbon and oxygen in nodules.

Isotope dilution ($^{13}CO_2$ enrichment) technique and measuring oxygen supply to nodules could be used to test whether the mixed strain infected nodules gain more carbon and oxygen compared to single strains infected nodules. Young and Matthews (1982) showed that there was strain specificity of Afghanistan pea cultivars to *R. leguminosarum.* Davis et al. (1988) further demonstrated some strains contained gene *nodX* (cultivar or genotype-specific nodulation gene) in their plasmids which were compatible only with primitive Afghanistan pea cultivars. Therefore, determining whether there is a variation among nod genes of *R. leguminosarum* strains and their compatibility with the *sym-2* gene (gene responsible for interacting with rhizobial *nodX* gene (Smith and Goodman, 1999) of cultivar Wharton and Twilight would reveal more evidence for this mixed strain infection scenario in root nodules. Thereby, beneficial symbiotic genes of cultivars could be exploited for new field pea breeding programs.

6.3.3. Production and application of drought tolerant inoculants to improve grain yield

This current work emphasized the need for producing drought tolerant, competitive commercial rhizobial inoculants which persist in legume fields providing more fixed N. Notably, when the commercial inoculant (WSM1455) was added with competitor strains, the efficiency of nodulation, N fixation, plant biomass and total N were significantly lowered compared to the plants with WSM1455 alone (chapter 4). The suggested hypothetical scenario was that WSM1455 was facing intense competitive pressure by competitors during symbiosis. This could be experimentally tested using antibiotic production assays by the competitor strains (Cole and Elkan, 1979) and if any competitor strain produces antibiotics then looking at whether WSM1455 could contain any antibiotic resistant genes. Moreover, WSM1455 could be incorporated with drought resistance genes to retain in water-stressed soils.

6.3.4 Exploiting beneficial NREs for improving legume N fixation

Identification and isolation of beneficial NREs to inoculate along with efficient rhizobial inoculants would enhance crop production. It can be recommended to repeat the experiments in chapter 5 to determine whether the outcomes are consistent under the same experimental conditions. Emerging studies have explored that soil micro organism networks and interactions among them are complex and dynamic (Wagg et al., 2019). The interactions of NRE between competitor strains and commercial inoculants might vary according to the type of strains present and the environmental conditions prevailing in the field. Therefore, there can be variation in microbial interactions through processes, such as competition, inhibition and symbiotic

association (Barberán et al., 2012). It has also been revealed that the complexity of these microbial interactions could be determined by the number of associations shared among them (Banerjee et al., 2019). Further evaluating the specific roles of NREs in root nodules would allow to predict their contribution to field pea growth and function. For example, the specific NRE strains could be isolated and re-inoculated separately or in combinations to determine their effect on plant growth and function. The roles of NRE in anti-pathogen activity could be determined by co-inoculating with a fungal or bacterial pathogen (Schwartz et al., 2013). It is also suggested that extracting anti-bacterial compounds (if any) in NRE isolates and testing them on rhizobia could reveal whether NREs are having any antagonistic effects (Bai et al., 2002). The outcomes of these biochemical tests could further provide evidence whether these three-party interactions between competitors, NREs and WSM1455 could influence NRE diversity in root nodules. Approaches quantifying the host's resources (carbon and oxygen) in root nodules would disclose whether the compositional shifts in NRE communities could be driven by plant driven sanctions. Collectively, these findings would provide more information on selecting beneficial NREs associated with particular legumes and their rhizobial symbionts.

6.4 Conclusion

Rhizobial N fixation provides an important source of nitrogen for legumes while partially replacing mineral nitrogen fertilizer inputs in agricultural systems. Success when introducing a commercial inoculant can be reduced by the environmental constraints of the field (e.g. drought) and by ineffective rhizobial populations (competitors). Therefore, a commercial rhizobial inoculant should be competitive in nodulation, efficient in N fixation and resistant for existing environmental constraints in the field. Multi-strain rhizobial inoculants can provide more symbiotic benefits for legumes over single-strain inoculants, but my work has highlighted that interactions among strains of rhizobia can be complex and require additional testing. In addition, NRE communities do interact with inoculated rhizobia under a variety of conditions, and these interactions may provide fruitful opportunities to improve N fixation outcomes for field pea and possibly other legumes.

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Appendix 1

Chapter 2- Supplementary material

Table S2- 1: Descriptions of *Rhizobium leguminosarum* strains obtained from DPI, Victoria including bioregion, associated crops and soil properties

STRAIN	ORIGIN	Country	GPS	PLANT	Soil	pH	Altitude
RRI 294	SITE 210 (BURRAM VIC)	Australia	6-54-429 E 59-47-010N				
RRI 429	Q SITE 1 Chitwan, 3-5km east Bharatpur	Nepal	None	Lentil	Clay Loam; gravel	(kit) 6.15	
RRI 510	Q SITE 8 10km from Bhaktapur, east of Kathmandu	Nepal	None	Lentil	Clay loam; organic	N/A	5000 (ft)
RRI 546	Q SITE 8 10km from Bhaktapur, east of Kathmandu	Nepal	None	Lentil	Clay loam; organic	N/A	5000 (ft)
RRI 548	Q SITE 8 10km from Bhaktapur, east of Kathmandu	Nepal	None	Lentil	Clay loam; organic	N/A	5000 (ft)
RRI 590	Q SITE 8 10km from Bhaktapur, east of Kathmandu	Nepal	None	Lentil	Clay loam; organic	N/A	5000 (ft)
RRI 607	Q SITE 8 10km from Bhaktapur, east of Kathmandu	Nepal	None	Lentil	Clay loam	N/A	3000 (ft)
RRI 610	Q SITE 8 10km from Bhaktapur, east of Kathmandu	Nepal	None	Lentil	Clay loam	N/A	3000 (ft)
RRI 613	Q SITE 8 10km from Bhaktapur, east of Kathmandu	Nepal	None	Lentil	Clay loam	N/A	3000 (ft)
RRI 970	Q SITE 18 Gallipoli Peninsula Turkey	Turkey	None	Vetch	Sandy loam; stoney		
RRI 1204	Q SITE 21 Bethloney Nepal	Nepal	28-01-072 N 081-42-472 E	Lentil	Clay loam	(h20) 6.89	117m
RRI 1220	Q SITE 21 Bethloney Nepal	Nepal	28-01-072 N 081-42-472 E	Vetch	Clay loam	(h20) 6.90	117m

Table S2- 2: N-limited Hogland's nutrient solution (Revised from Somasegaran and Hoben, 1985)

Component	Stock Solution	mL Stock Solution/1 L
Macronutrients		
1M $CaCl_2$	146 g/L	2
1M KH_2PO_4	6.8g/L	1
2M $MgSO_4.7H2O$	12.3 g/L	2
0.5M KNO_3	3.73g/L	1
Micronutrients		
H_3BO_3	2.86 g/L	1
MnO_4S	1.81 g/L	1
$ZnSO_4.7H2O$	0.22 g/L	1
$CuSO_4.5H2O$	0.08 g/L	1
$H_2MoO_4.H2O$ or	0.09 g/L	1
Iron		
0.5% $C_6H_5FeO_7$	136 g/L	1

Note: A small concentration of KNO_3 was added to maintain plant growth

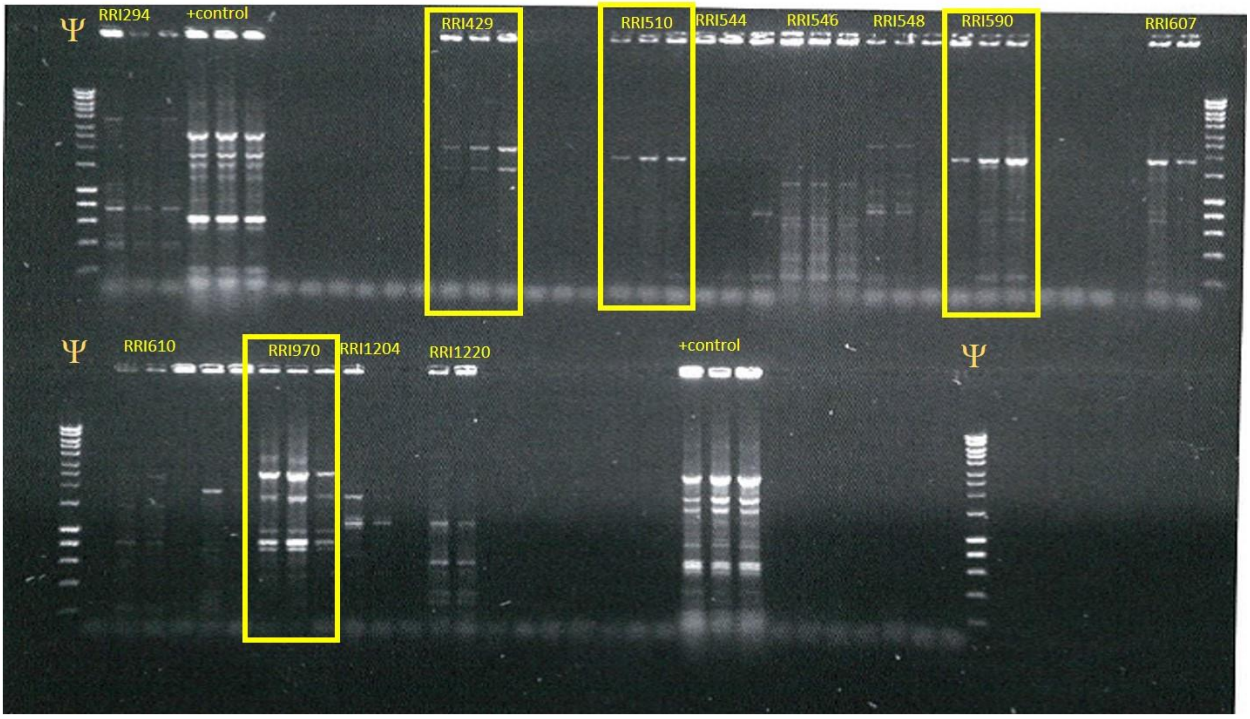

Figure S2- 1: Agarose gel image of ERIC fingerprints of 12 Rhizobium leguminosarum isolates. Three replicate PCRs for each isolate are run adjacently to each other. Ψ is a 1KB marker (Bioline, AU). Yellow boxes highlight examples of unique profiles of two different isolates RRI429, RRI510, RRI590 and RRI970 respectively.

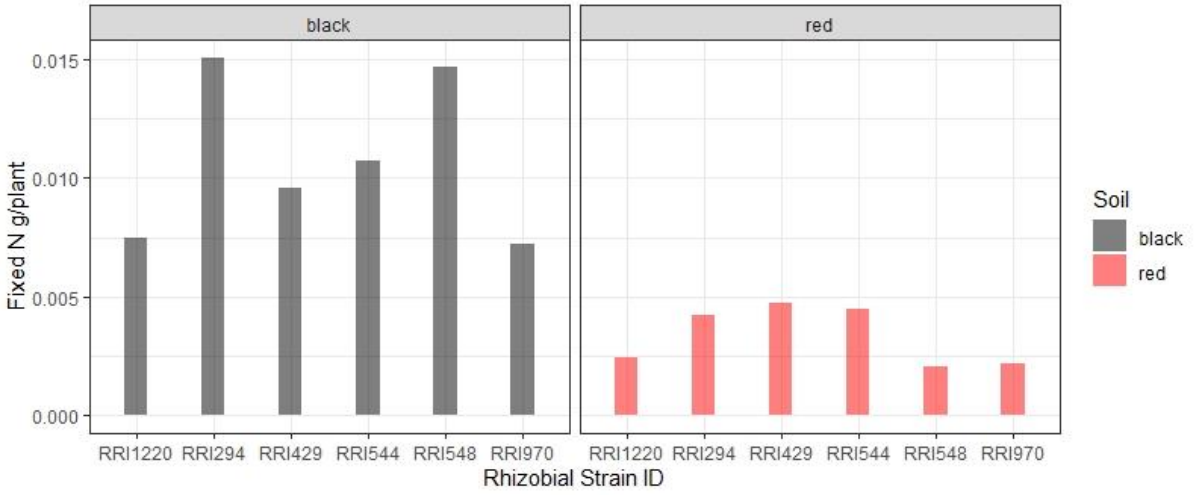

Figure S2- 2: Amounts of fixed nitrogen (N) per plant inoculated with monocultures of *Rhizobium leguminosarum* RRI strains. Responses were measured for plants grown in two different soils: a black vertosol ('black') and a red calcarosol ('red'). Results shown are for n=4 (biological replicates). *P*= 0.02 and 0.14 in black and red soils respectively (One-way ANOVA Kenward Rodger DF)

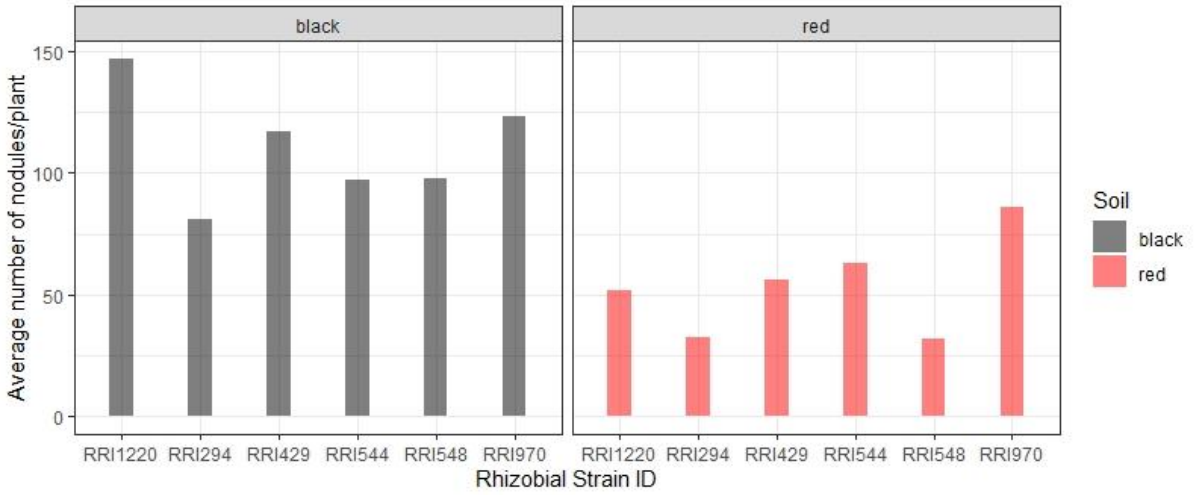

Figure S2- 3: Average number of nodules per plant inoculated with monocultures of Rhizobium leguminosarum RRI strains. Responses were measured for plants grown in two **different soils: a black** vertosol ('black') and a red calcarosol ('red'). Results shown are for n=4 (biological replicates).

Table S2- 3: Processed data for single RRI strain inoculation experiment including average number of nodules per plant, dN15, d13C,% NDFA, Total N and fixed amount of N per plant

Treatment	Isolate	Soil	Average dN15	Average dN13C	Average nodules	%Ndfa	Average TotN_g	FixedN_g
monoculture	RRI1220	black	3.28	-28.86	146.75	49.03	0.017	0.007
monoculture	RRI1220	red	1.49	-28.88	51.75	65.65	0.005	0.002
monoculture	RRI294	black	1.18	-29.2	81.25	81.68	0.019	0.015
monoculture	RRI294	red	1.43	-29.55	32.5	66.97	0.006	0.004
monoculture	RRI429	black	1.32	-29.93	117.25	79.50	0.012	0.01
monoculture	RRI429	red	1.58	-30.16	56	63.45	0.008	0.005
monoculture	RRI544	black	1.61	-29.12	97.25	74.96	0.015	0.011
monoculture	RRI544	red	2.06	-29.98	63	58.31	0.008	0.005
monoculture	RRI548	black	0.79	-30.13	98	87.73	0.017	0.015
monoculture	RRI548	red	2.21	-29.21	32	36.78	0.007	0.002
monoculture	RRI970	black	3.14	-29.54	123	51.32	0.014	0.007
monoculture	RRI970	red	2.17	-29.56	86.25	49.88	0.005	0.002

Table S2- 4: dN15 values plant shoots inoculated with *Rhizobium leguminosarum* RRI strain combinations (belong to both low and high genetic similarity groups), uninoculated field pea plants grown in red soil black soil and N-free vermiculite substrate.

Treatment	isolate	soil	d15N
Low similarity	RRI294/RRI1220	black	1.58
Low similarity	RRI294/RRI1220	black	2.17
Low similarity	RRI294/RRI1220	black	3.48
Low similarity	RRI294/RRI1220	black	1.01
Low similarity	RRI294/RRI1220	red	2.55
Low similarity	RRI294/RRI1220	red	2.48
Low similarity	RRI294/RRI1220	red	2.78
Low similarity	RRI294/RRI1220	red	2.18
High similarity	RRI294/RRI429	black	1.05
High similarity	RRI294/RRI429	black	1.07
High similarity	RRI294/RRI429	black	0.94
High similarity	RRI294/RRI429	black	1.47
High similarity	RRI294/RRI429	red	0.43
High similarity	RRI294/RRI429	red	0.99
High similarity	RRI294/RRI429	red	0.42
High similarity	RRI294/RRI429	red	0.11
High similarity	RRI294/RRI970	black	2.62
High similarity	RRI294/RRI970	black	0.94
High similarity	RRI294/RRI970	black	2.86
High similarity	RRI294/RRI970	black	2.14
High similarity	RRI294/RRI970	red	2.78
High similarity	RRI294/RRI970	red	1.93
High similarity	RRI294/RRI970	red	1.5
High similarity	RRI294/RRI970	red	1.87
Low similarity	RRI429/RRI1220	black	-0.34
Low similarity	RRI429/RRI1220	black	3.45
Low similarity	RRI429/RRI1220	black	0.72
Low similarity	RRI429/RRI1220	black	0.71
Low similarity	RRI429/RRI1220	red	1.31
Low similarity	RRI429/RRI1220	red	0.78
Low similarity	RRI429/RRI1220	red	2.36
Low similarity	RRI429/RRI1220	red	-0.05

High similarity	RRI548/RRI970	black	0.61
High similarity	RRI548/RRI970	black	2.72
High similarity	RRI548/RRI970	black	1.24
High similarity	RRI548/RRI970	black	1.49
High similarity	RRI548/RRI970	red	2.48
High similarity	RRI548/RRI970	red	1.03
High similarity	RRI548/RRI970	red	2.02
High similarity	RRI548/RRI970	red	1.65
Low similarity	RRI970/RRI1220	black	2.78
Low similarity	RRI970/RRI1220	black	1.2
Low similarity	RRI970/RRI1220	black	0.48
Low similarity	RRI970/RRI1220	black	1.61
Low similarity	RRI970/RRI1220	red	3.19
Low similarity	RRI970/RRI1220	red	3.06
Low similarity	RRI970/RRI1220	red	3.51
Low similarity	RRI970/RRI1220	red	0.87
High similarity	RRI970/RRI429	black	0.74
High similarity	RRI970/RRI429	black	1.49
High similarity	RRI970/RRI429	black	0.98
High similarity	RRI970/RRI429	black	4.46
High similarity	RRI970/RRI429	red	0.96
High similarity	RRI970/RRI429	red	-0.75
High similarity	RRI970/RRI429	red	1.04
High similarity	RRI970/RRI429	red	3.63
NA	uninoculated	black	2.72
NA	uninoculated	black	2.87
NA	uninoculated	black	0.25
NA	uninoculated	red	0.04
NA	uninoculated	red	1.9
NA	uninoculated	red	0.9
NA	uninoculated	vermiculite	-0.15
NA	uninoculated	vermiculite	0.33
NA	uninoculated	vermiculite	1.46

NA	uninoculated	vermiculite	2.25
NA	uninoculated	vermiculite	-0.51
NA	uninoculated	vermiculite	2.44
NA	uninoculated	vermiculite	-0.23
NA	uninoculated	vermiculite	NA
NA	uninoculated	vermiculite	0.65
NA	uninoculated	vermiculite	1.72

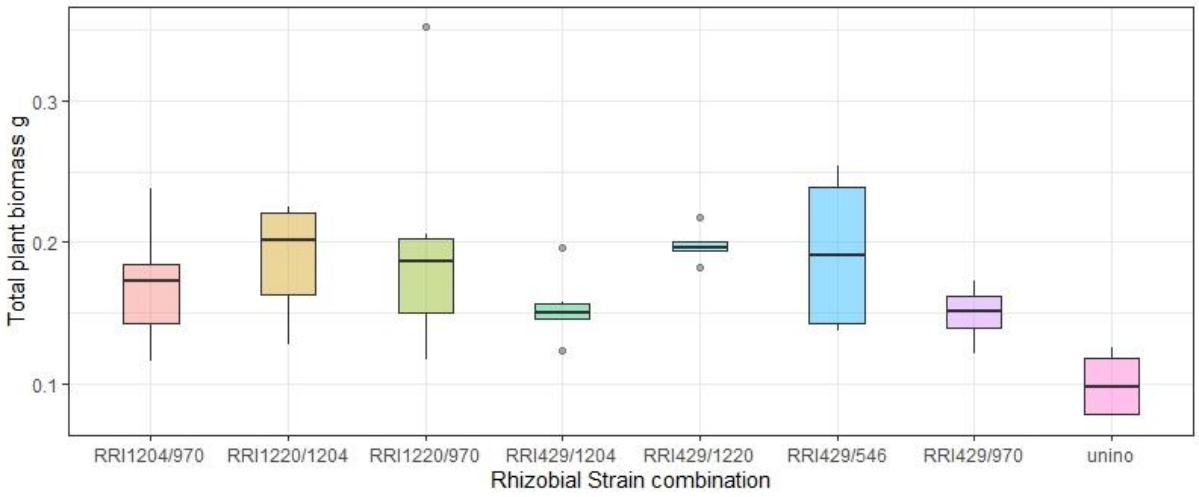

Figure S2- 4: Total plant biomass of pea plants co-inoculated with pairs of Rhizobium leguminosarum strains and uninoculated pea plants. Results shown are for n=6 (biological replicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range. $P_{\text{Rhizobial inoculation}}$ was 0.04 (one-way ANOVA (Type II Wald F tests with Kenward-Roger DF)).

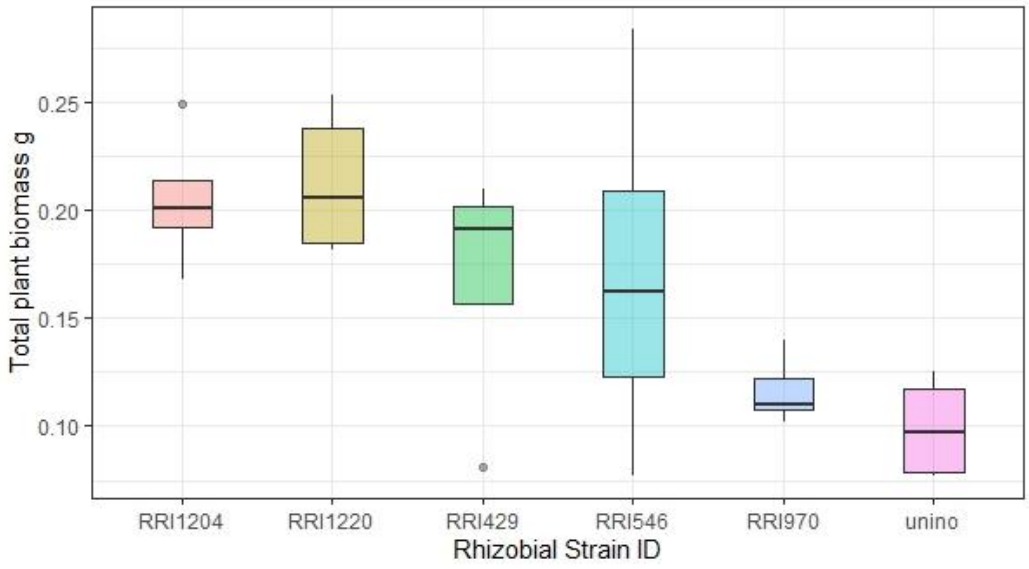

Figure S2- 5: Total plant biomass of pea plants inoculated with single strains of *Rhizobium leguminosarum* and plants those were uninoculated. Results shown are for n=6 (biological replicates). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range and when dots are present, extreme values within 1.5 times the interquartile range. $P_{\text{Rhizobial inoculation}}$ was 0.006 (one-way ANOVA (Type II Wald F tests with Kenward-Roger DF)).

Appendix 2

Chapter 3- Supplementary material

Figure S3- 1: Emergence of root nodules in pea seedlings after rhizobial inoculations

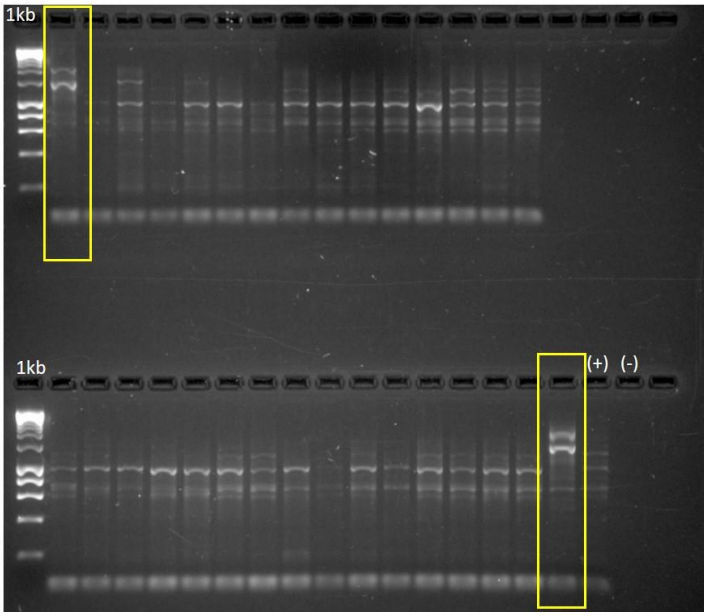

Figure S3- 2: Agarose gel image of ERIC fingerprints of Rhizobium *leguminosarum* isolates RRI429 and RRI546. 1kb is the marker (Bioline, AU). Yellow boxes highlight examples of unique banding profile belong to RRI429 and rest of the banding profiles are with close similarity with RRI546. (+) control was a soil DNA extract and (-) was the PCR master mix without DNA.

Appendix 3

Chapter 4-Supplementary Material

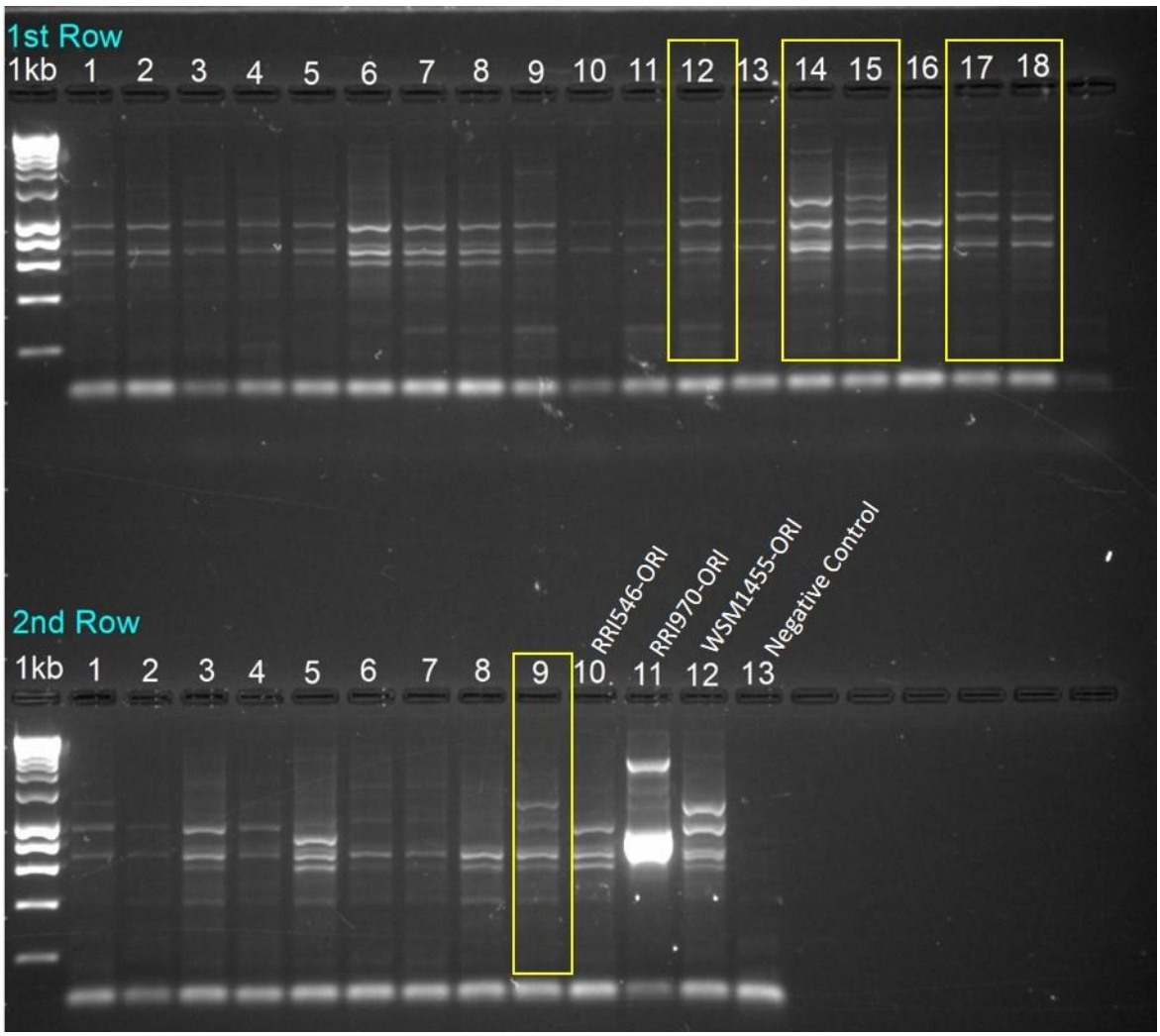

Figure S4- 1: Agarose gel image of ERIC PCR fingerprints belong to 27 individual root nodule DNA extracts. 1kb is the marker (Bioline, Australia). Wells from10-12 show DNA of original isolates belong to RRI546, RRI970 and WSM1455 respectively. Negative control contained ERIC PCR master mix without any DNA (well 13). Yellow highlighted boxes indicate unique banding patterns belong to WSM1455. The remaining banding patterns (except controls and yellow highlights) closely resemble bands of RRI546.

Plots indicating the main-effects of rhizobial inoculation type, watering condition and pea cultivar on plant responses

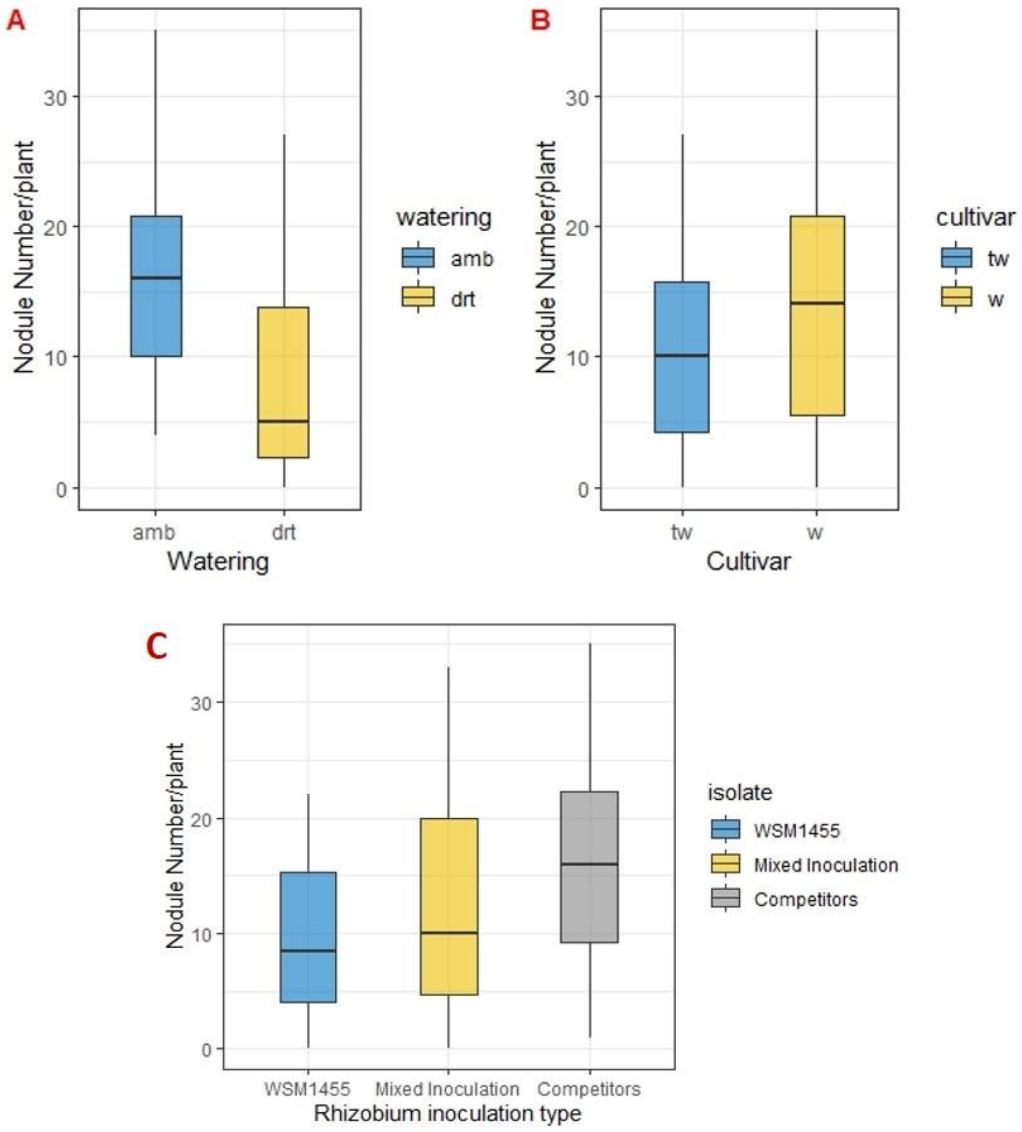

Figure S4- 2: Total nodule number of field pea plants averaged A) under well- watered (amb) and drought (drt) conditions, B) in two different cultivars; Twilight (tw) and Wharton (w) and C) inoculated with three rhizobial inoculation types (WSM1455, Mixed inoculation & Competitors). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

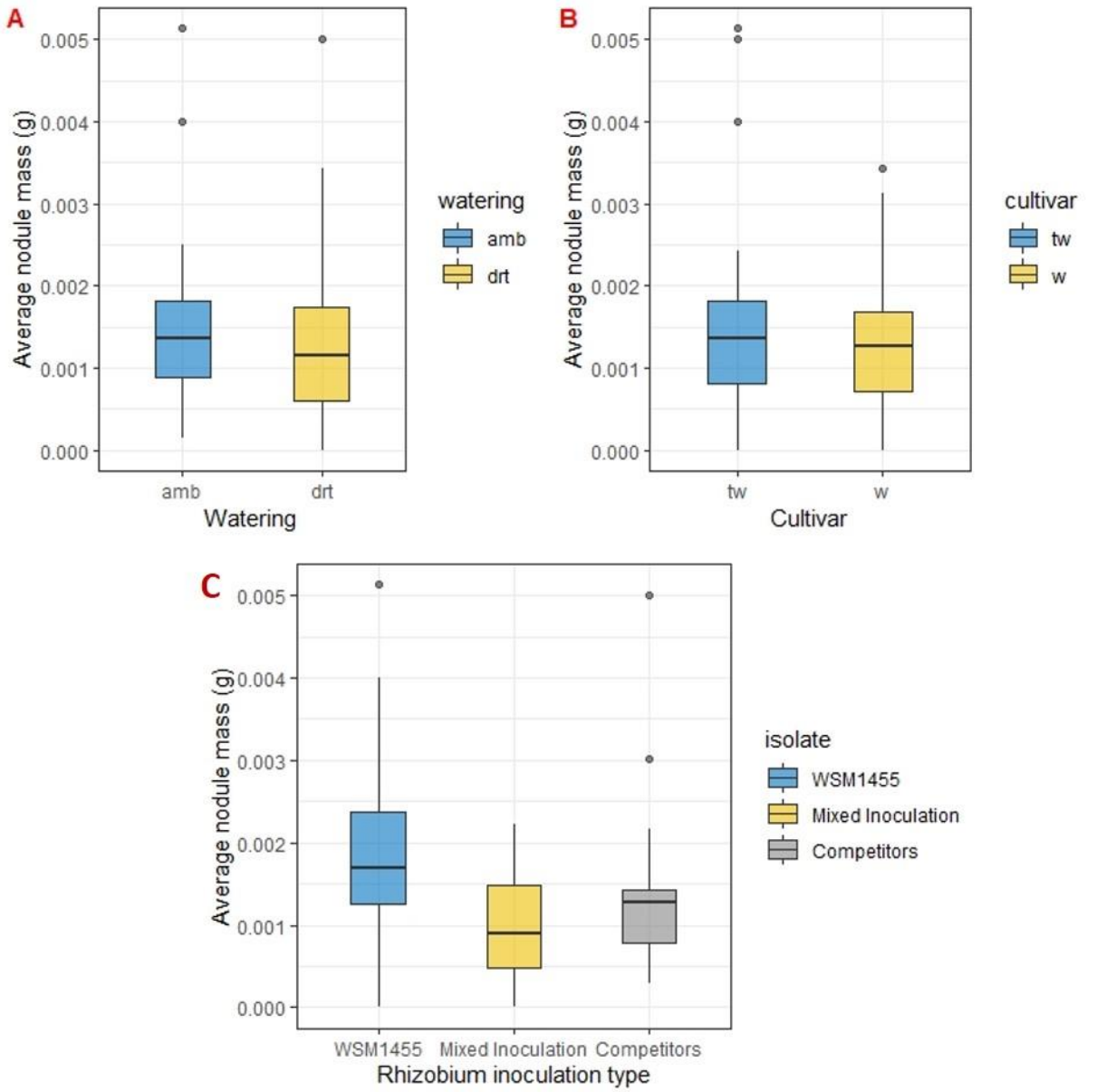

Figure S4- 3: Average nodule mass of field pea plants averaged A) under well- watered (amb) and drought (drt) conditions, B) in two different cultivars; Twilight (tw) and Wharton (w) and C) inoculated with three rhizobial inoculation types (WSM1455, Mixed inoculation & Competitors). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

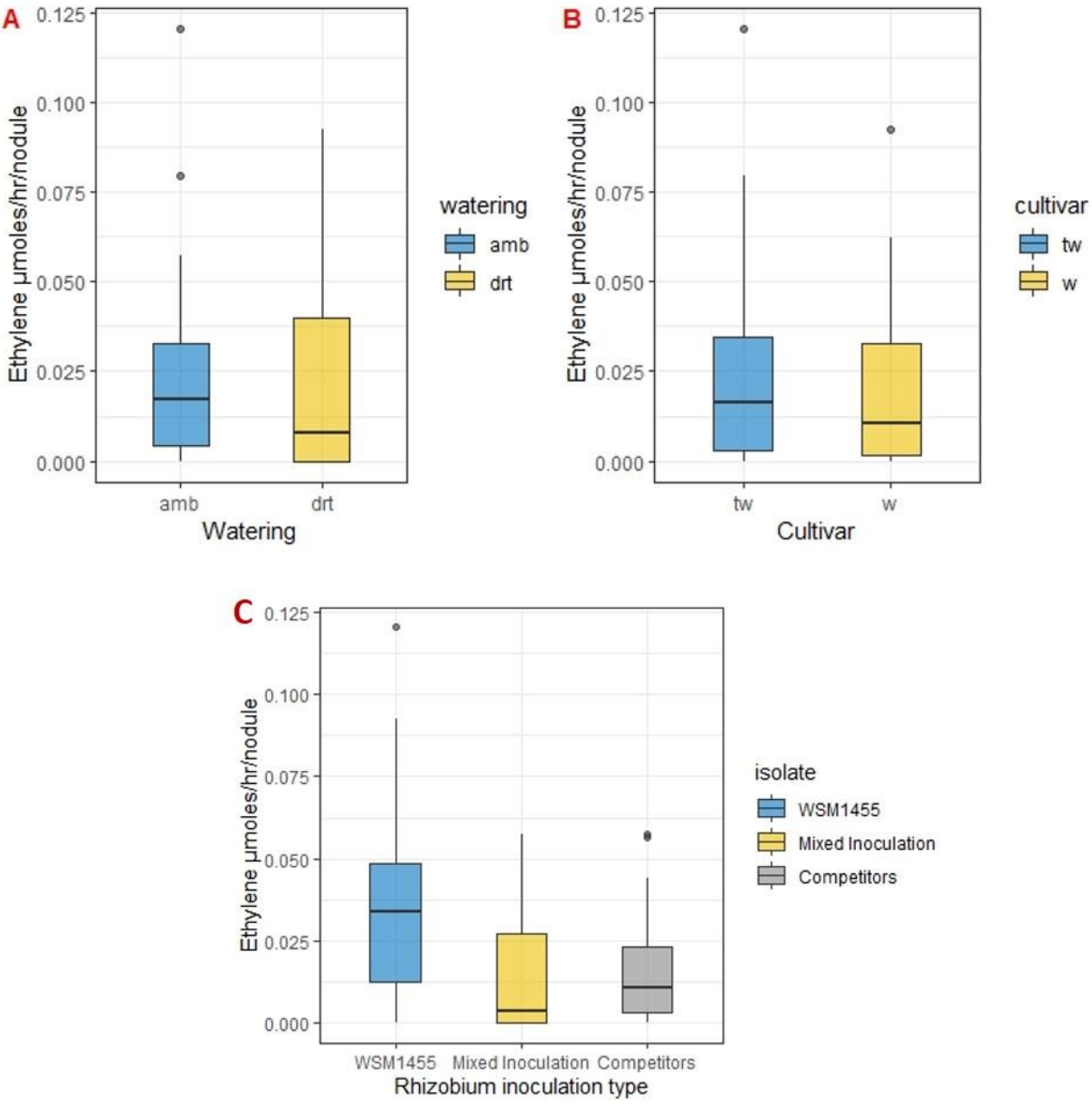

Figure S4- 4: Nodule level nitrogen fixation efficiency of field pea plants averaged A) under well- watered (amb) and drought (drt) conditions, B) in two different cultivars; Twilight (tw) and Wharton (w) and C) inoculated with three rhizobial inoculation types (WSM1455, Mixed inoculation & Competitors). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

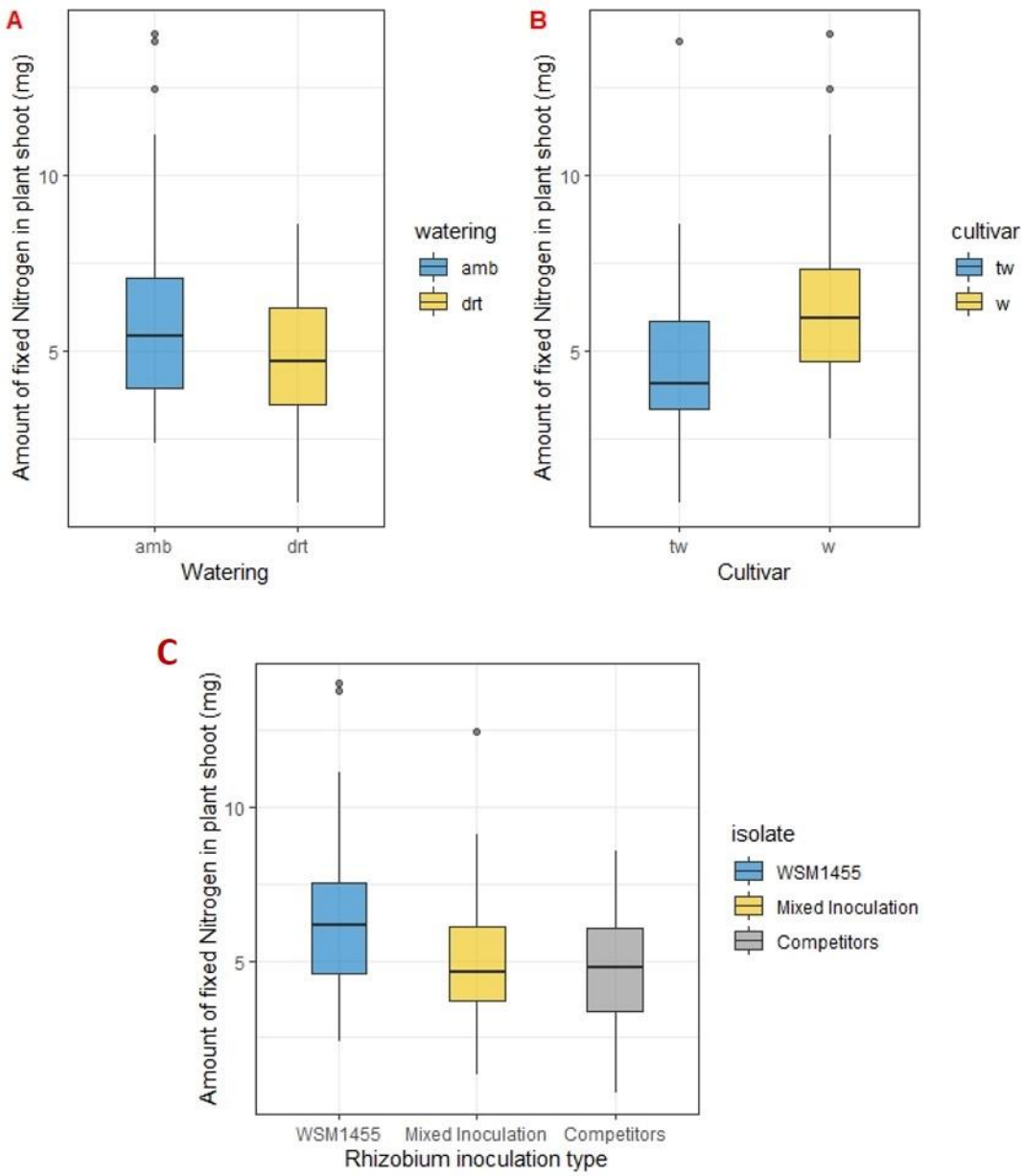

Figure S4- 5: The total amount of fixed N in shoot tissues of field pea plants averaged A) under well- watered (amb) and drought (drt) conditions, B) in two different cultivars; Twilight (tw) and Wharton (w) and C) inoculated with three rhizobial inoculation types (WSM1455, Mixed inoculation & Competitors). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

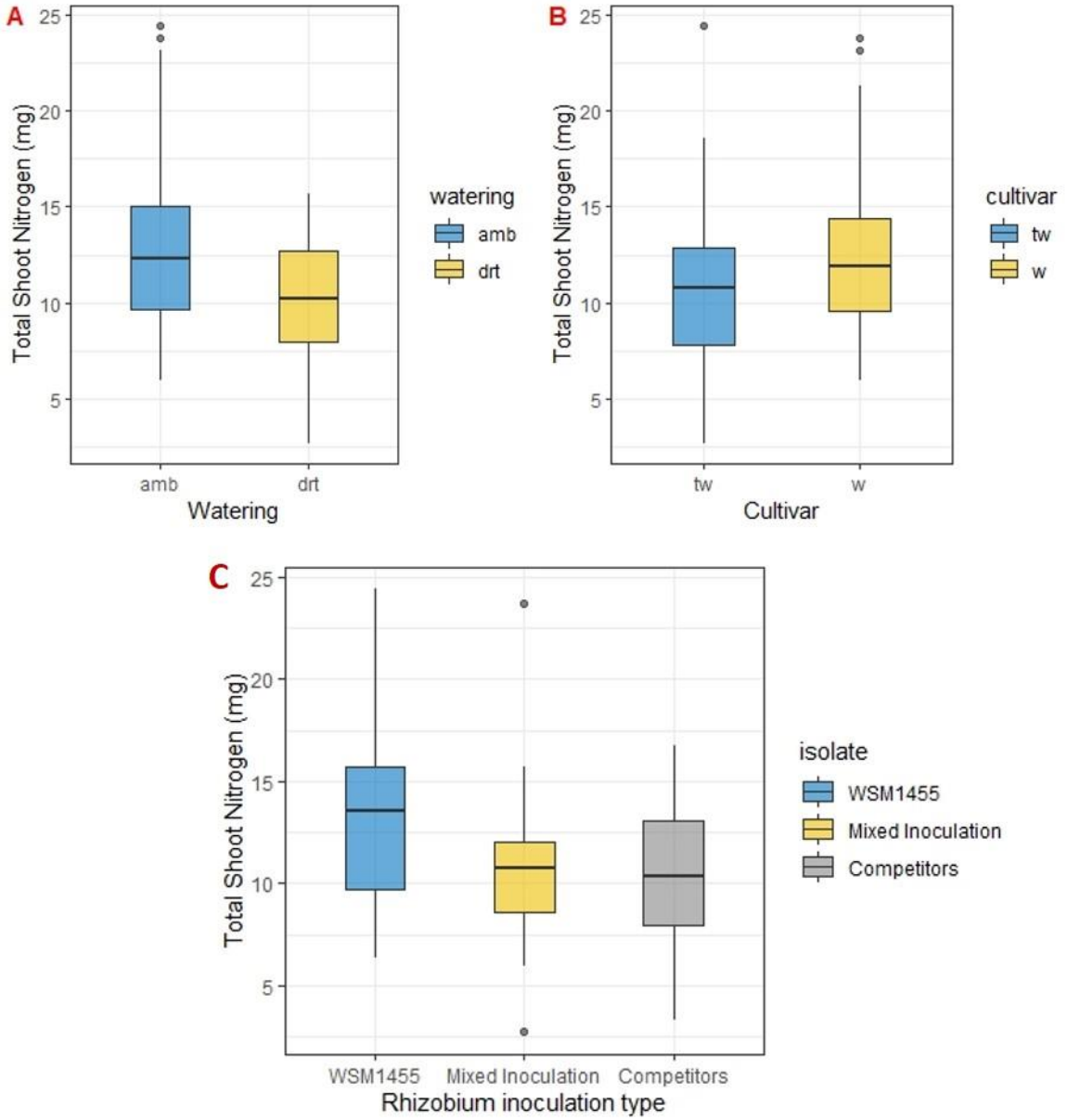

Figure S4- 6: Total plant (shoot) nitrogen content of field pea plants averaged A) under well- watered (amb) and drought (drt) conditions, B) in two different cultivars; Twilight (tw) and Wharton (w) and C) inoculated with three rhizobial inoculation types (WSM1455, Mixed inoculation & Competitors). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

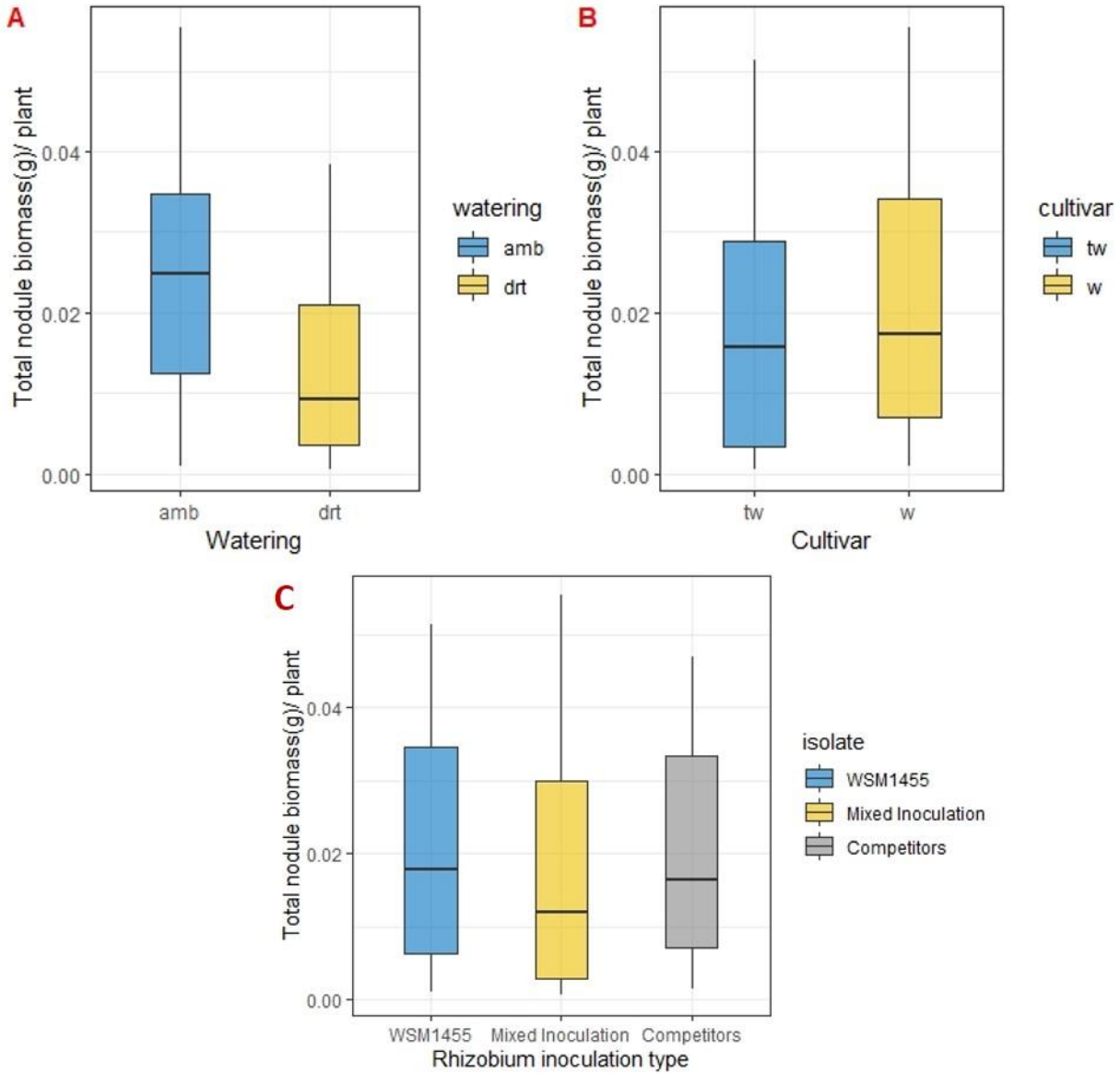

Figure S4- 7: Total nodule biomass of field pea plants averaged A) under well- watered (amb) and drought (drt) conditions, B) in two different cultivars; Twilight (tw) and Wharton (w) and C) inoculated with three rhizobial inoculation types (WSM1455, Mixed inoculation & Competitors). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

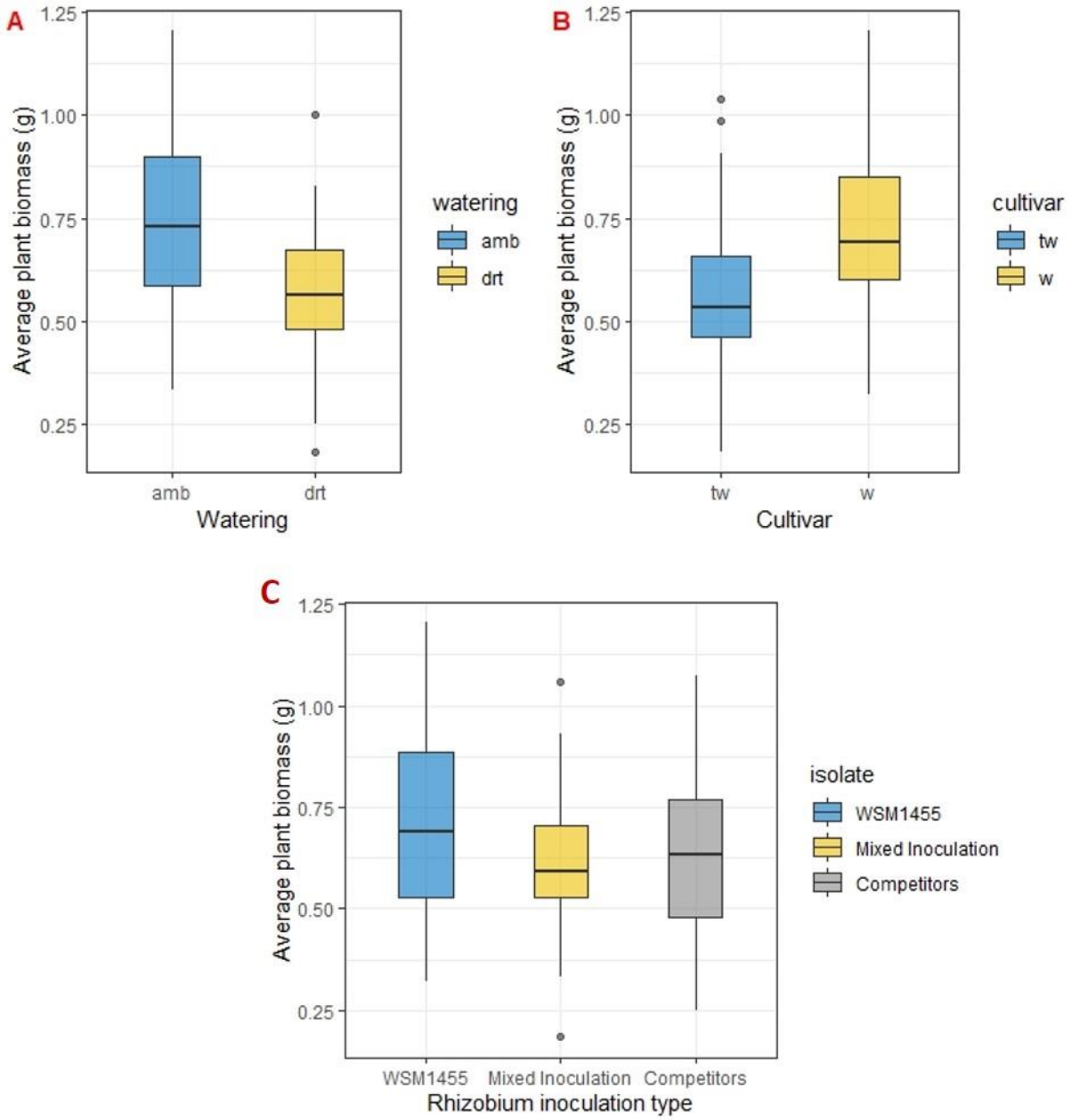

Figure S4- 8: Average total biomass of field pea plants averaged A) under well- watered (amb) and drought (drt) conditions, B) in two different cultivars; Twilight (tw) and Wharton (w) and C) inoculated with three rhizobial inoculation types (WSM1455, Mixed inoculation & Competitors). The black horizontal line inside the box indicates the median value, the bottom and top borders of the box represent the first and third quartiles and the vertical lines represent the range or, when dots are present, extreme values within 1.5 times the interquartile range.

Table S4- 1:Summary of two-way analysis of variance (ANOVA) between cultivar and watering condition on total plant biomass of un-inoculated pea plants

Source of variation	Total plant biomass		
	DF	**F**	***P***
Cultivar	1,8	0.38	0.55
Watering	1,8	0.11	0.74
Watering x Cultivar	1,8	0.14	0.71

Table S4- 2: Data table for δ15N, shoot, root and total plant biomass, total amount of shoot nitrogen and total amount of fixed nitrogen in shoot for inoculated (WSM1455, RRI546+RRI970 and mixed inoculation) and uninoculated field pea plants grown under ambient watering and reduced watering treatments

ID	watering	cultivar	isolate	shoot mass g	root mass g	Total biomass g	d15N	N Amount (ug)	N amount (mg)	shoot amount used (mg)	TotN shoot mg	%Ndfa	fixedN mg
250	amb	tw	WSM1455	0.54	0.07	0.61	6.24	75.51	0.08	4.35	9.42	25.21	2.37
251	amb	tw	WSM1455	0.82	0.16	0.99	5.45	86.73	0.09	4.27	16.75	36.64	6.13
252	amb	tw	WSM1455	0.50	0.14	0.64	6	90.26	0.09	4.88	9.19	28.74	2.64
253	amb	tw	WSM1455	0.29	0.16	0.46	3.86	139.98	0.14	5.26	7.74	59.57	4.61
254	amb	tw	WSM1455	0.54	0.19	0.73	5.16	134.01	0.13	5.14	14.12	40.84	5.77
255	amb	tw	WSM1455	0.87	0.17	1.04	4.07	130.48	0.13	4.64	24.39	56.50	13.78
256	amb	tw	WSM1455	0.72	0.18	0.91	5.26	122.49	0.12	4.77	18.59	39.33	7.31
257	amb	w	WSM1455	0.93	0.27	1.20	3.41	94.86	0.09	4.17	21.26	52.34	11.13
258	amb	w	WSM1455	0.64	0.20	0.84	3.97	104.71	0.1	4.46	15.02	45.95	6.90
259	amb	w	WSM1455	0.60	0.16	0.76	5.14	105.29	0.11	4.48	14.06	32.59	4.58
260	amb	w	WSM1455	0.71	0.17	0.88	3.43	112.66	0.11	3.99	19.93	52.08	10.38
261	amb	w	WSM1455	0.88	0.20	1.08	3.34	87.62	0.09	4.41	17.51	53.10	9.30
262	amb	w	WSM1455	0.57	0.12	0.69	5.13	109.24	0.11	4.09	15.34	32.69	5.01
263	amb	w	WSM1455	0.81	0.23	1.04	2.68	123.75	0.12	4.31	23.13	60.63	14.02
264	drt	tw	WSM1455	0.42	0.11	0.52	4.1	116.48	0.12	3.81	12.80	50.96	6.52
265	drt	tw	WSM1455	0.29	0.14	0.43	4.45	110.29	0.11	4.26	7.56	46.57	3.52
266	drt	tw	WSM1455	0.31	0.03	0.34	3.92	95.55	0.1	4.62	6.37	53.17	3.39
267	drt	tw	WSM1455	0.43	0.10	0.53	5.34	109.63	0.11	4.01	11.68	35.29	4.12
268	drt	tw	WSM1455	0.37	0.11	0.48	3.98	128.9	0.13	4.24	11.23	52.50	5.90
269	drt	tw	WSM1455	0.35	0.21	0.57	4.58	118.26	0.12	4.12	10.12	44.87	4.54
270	drt	tw	WSM1455	0.34	0.15	0.48	4.85	101.65	0.1	4.21	8.14	41.46	3.37
271	drt	w	WSM1455	0.59	0.23	0.82	3.46	102.51	0.1	4.36	13.76	55.86	7.68
272	drt	w	WSM1455	0.40	0.18	0.58	4.32	142.53	0.14	4.21	13.55	45.61	6.18
273	drt	w	WSM1455	0.83	0.17	1.00	4.06	68.08	0.07	4.19	13.51	48.66	6.57
274	drt	w	WSM1455	0.53	0.15	0.68	3.79	107.17	0.11	3.93	14.46	51.93	7.51
275	drt	w	WSM1455	0.24	0.08	0.32	1.37	127.9	0.13	4.02	7.73	80.90	6.25
276	drt	w	WSM1455	0.69	0.11	0.80	3.26	84.81	0.08	4.42	13.16	58.31	7.67
277	drt	w	WSM1455	0.51	0.16	0.67	4.12	77.63	0.08	4.01	9.78	48.04	4.70

278	amb	tw	Competitors	0.69	0.12	0.82	4.53	86.71	0.09	4.09	14.70	48.58	7.14
279	amb	tw	Competitors	0.51	0.10	0.61	6.1	97.53	0.1	4.19	11.80	26.56	3.13
280	amb	tw	Competitors	0.28	0.07	0.35	3.45	104.37	0.1	4.14	7.18	63.69	4.57
281	amb	tw	Competitors	0.41	0.07	0.48	6.13	109.43	0.11	4.37	10.24	26.03	2.67
282	amb	tw	Competitors	0.53	0.13	0.66	5.49	90.08	0.09	4.31	11.01	35.03	3.86
283	amb	tw	Competitors	0.66	0.10	0.76	4.84	85.73	0.09	4.18	13.47	44.19	5.95
284	amb	tw	Competitors	0.59	0.14	0.72	5.23	85.8	0.09	3.89	12.94	38.69	5.01
285	amb	w	Competitors	0.60	0.25	0.85	3.41	62.17	0.06	4.15	9.07	53.15	4.82
286	amb	w	Competitors	0.75	0.24	0.99	3.5	61.25	0.06	3.80	12.10	52.13	6.31
287	amb	w	Competitors	0.72	0.13	0.85	5.34	73.52	0.07	4.05	13.05	30.76	4.01
288	amb	w	Competitors	0.79	0.20	0.99	4.27	80.26	0.08	3.81	16.71	43.18	7.21
289	amb	w	Competitors	0.77	0.31	1.07	4.31	82.29	0.08	4.19	15.07	42.77	6.45
290	amb	w	Competitors	0.29	0.09	0.38	1.74	109.49	0.11	4.05	7.84	72.67	5.69
291	amb	w	Competitors	0.67	0.13	0.80	3.52	81.11	0.08	4.33	12.64	51.91	6.56
292	drt	tw	Competitors	0.15	0.14	0.29	6.53	100.27	0.1	4.57	3.30	20.37	0.67
293	drt	tw	Competitors	0.18	0.15	0.33	3	111.89	0.11	3.83	5.12	65.25	3.34
294	drt	tw	Competitors	0.17	0.08	0.25	5.16	113.53	0.11	3.89	5.00	37.77	1.89
295	drt	tw	Competitors	0.38	0.18	0.56	4.19	141.23	0.14	3.87	13.83	50.14	6.93
296	drt	tw	Competitors	0.47	0.06	0.52	3.54	117.18	0.12	3.73	14.69	58.35	8.57
297	drt	tw	Competitors	0.33	0.16	0.48	4.41	93.42	0.09	3.93	7.72	47.24	3.65
298	drt	tw	Competitors	0.18	0.13	0.30	3.54	124.86	0.12	4.17	5.28	58.29	3.08
299	drt	w	Competitors	0.56	0.12	0.68	4.89	92.53	0.09	3.93	13.24	38.79	5.14
300	drt	w	Competitors	0.36	0.21	0.56	4.36	116.85	0.12	4.05	10.24	45.16	4.62
301	drt	w	Competitors	0.58	0.11	0.69	3.01	70.01	0.07	4.27	9.43	61.32	5.78
302	drt	w	Competitors	0.40	0.08	0.47	4.64	78.96	0.08	3.92	7.97	41.81	3.33
303	drt	w	Competitors	0.51	0.17	0.67	3.69	86.78	0.09	4.17	10.52	53.26	5.60
304	drt	w	Competitors	0.51	0.16	0.67	3.97	83.11	0.08	4.46	9.55	49.87	4.76
305	drt	w	Competitors	0.36	0.20	0.56	5.63	101.78	0.1	4.38	8.29	30.00	2.49
306	amb	tw	Mixed Inoculation	0.56	0.22	0.77	4.95	90.75	0.09	4.41	11.43	41.83	4.78
307	amb	tw	Mixed Inoculation	0.50	0.16	0.66	6.01	124.45	0.12	4.41	14.07	27.18	3.82
308	amb	tw	Mixed Inoculation	0.22	0.11	0.33	3.11	115.84	0.12	4.29	5.98	67.11	4.01
309	amb	tw	Mixed Inoculation	0.52	0.17	0.70	5.5	89.48	0.09	4.07	11.49	34.26	3.94

310	amb	tw	Mixed Inoculation	0.37	0.20	0.57	2.74	98.73	0.1	4.05	8.93	72.29	6.45
311	amb	tw	Mixed Inoculation	0.43	0.15	0.58	6.54	122.99	0.12	4.24	12.59	19.89	2.50
312	amb	tw	Mixed Inoculation	0.26	0.17	0.43	4.68	131.76	0.13	4.20	8.18	45.53	3.73
313	amb	w	Mixed Inoculation	0.35	0.26	0.61	1.37	141.01	0.14	4.22	11.60	78.45	9.10
314	amb	w	Mixed Inoculation	0.83	0.23	1.06	3.57	120.85	0.12	4.23	23.74	52.43	12.45
315	amb	w	Mixed Inoculation	0.46	0.15	0.61	4.04	92.04	0.09	3.85	11.03	46.82	5.17
316	amb	w	Mixed Inoculation	0.41	0.10	0.51	2.17	111.04	0.11	4.27	10.71	68.98	7.39
317	amb	w	Mixed Inoculation	0.67	0.27	0.93	3.65	71.22	0.07	4.03	11.75	51.45	6.05
318	amb	w	Mixed Inoculation	0.36	0.16	0.52	4.25	85.05	0.09	3.84	7.99	44.27	3.54
319	amb	w	Mixed Inoculation	0.37	0.16	0.53	5.45	87.89	0.09	3.73	8.61	30.14	2.59
320	drt	tw	Mixed Inoculation	0.65	0.18	0.83	3.93	67.87	0.07	3.66	11.98	53.17	6.37
321	drt	tw	Mixed Inoculation	0.12	0.07	0.18	4.35	100.25	0.1	4.29	2.71	47.90	1.30
322	drt	tw	Mixed Inoculation	0.41	0.10	0.51	4.76	103.16	0.1	3.98	10.51	42.73	4.49
323	drt	tw	Mixed Inoculation	0.41	0.11	0.52	4.37	91.51	0.09	3.81	9.93	47.63	4.73
324	drt	tw	Mixed Inoculation	0.44	0.14	0.59	6.59	118.38	0.12	3.88	13.46	19.58	2.63
325	drt	tw	Mixed Inoculation	0.43	0.09	0.53	4.78	73.59	0.07	3.94	8.12	42.44	3.45
326	drt	tw	Mixed Inoculation	0.42	0.11	0.53	4.55	112.69	0.11	3.97	12.02	45.28	5.44
327	drt	w	Mixed Inoculation	0.60	0.22	0.82	3.88	87.03	0.09	4.12	12.59	49.74	6.26
328	drt	w	Mixed Inoculation	0.35	0.25	0.60	2.8	103.71	0.1	4.22	8.48	62.29	5.28
329	drt	w	Mixed Inoculation	0.40	0.25	0.65	3.61	117.66	0.12	4.53	10.32	52.90	5.46
330	drt	w	Mixed Inoculation	0.40	0.24	0.65	4.62	105	0.11	4.22	10.08	41.07	4.14
331	drt	w	Mixed Inoculation	0.31	0.26	0.57	2.9	80.89	0.08	4.18	5.99	61.16	3.66
332	drt	w	Mixed Inoculation	0.60	0.18	0.78	4	107.4	0.11	4.10	15.71	48.33	7.59
333	drt	w	Mixed Inoculation	0.53	0.20	0.73	4.93	78.34	0.08	3.88	10.70	37.40	4.00
334	amb	tw	UN	0.84	0.19	1.02	NA	89.9	0.09	4.04	18.62	NA	NA
335	amb	tw	UN	0.42	0.10	0.52	NA	88.26	0.09	4.30	8.57	NA	NA
336	amb	tw	UN	0.24	0.12	0.36	NA	126.58	0.13	4.28	7.14	NA	NA
337	amb	w	UN	0.47	0.20	0.67	NA	68.35	0.07	4.17	7.70	NA	NA
338	amb	w	UN	0.42	0.10	0.52	NA	105.4	0.11	3.82	11.59	NA	NA
339	amb	w	UN	0.56	0.24	0.80	NA	56.02	0.06	4.01	7.79	NA	NA
340	drt	tw	UN	0.60	0.17	0.77	NA	109.57	0.11	4.24	15.43	NA	NA
341	drt	tw	UN	0.33	0.08	0.41	NA	108.88	0.11	3.97	8.94	NA	NA

342	drt	tw	UN	0.37	0.08	0.45	NA	91.14	0.09	4.30	7.88	NA	NA
343	drt	w	UN	0.71	0.12	0.83	NA	73.77	0.07	3.77	13.99	NA	NA
344	drt	w	UN	0.35	0.16	0.51	NA	106.04	0.11	3.95	9.39	NA	NA
345	drt	w	UN	0.50	0.17	0.67	NA	67.17	0.07	3.84	8.71	NA	NA

Appendix 4

Chapter 5- Supplementary material

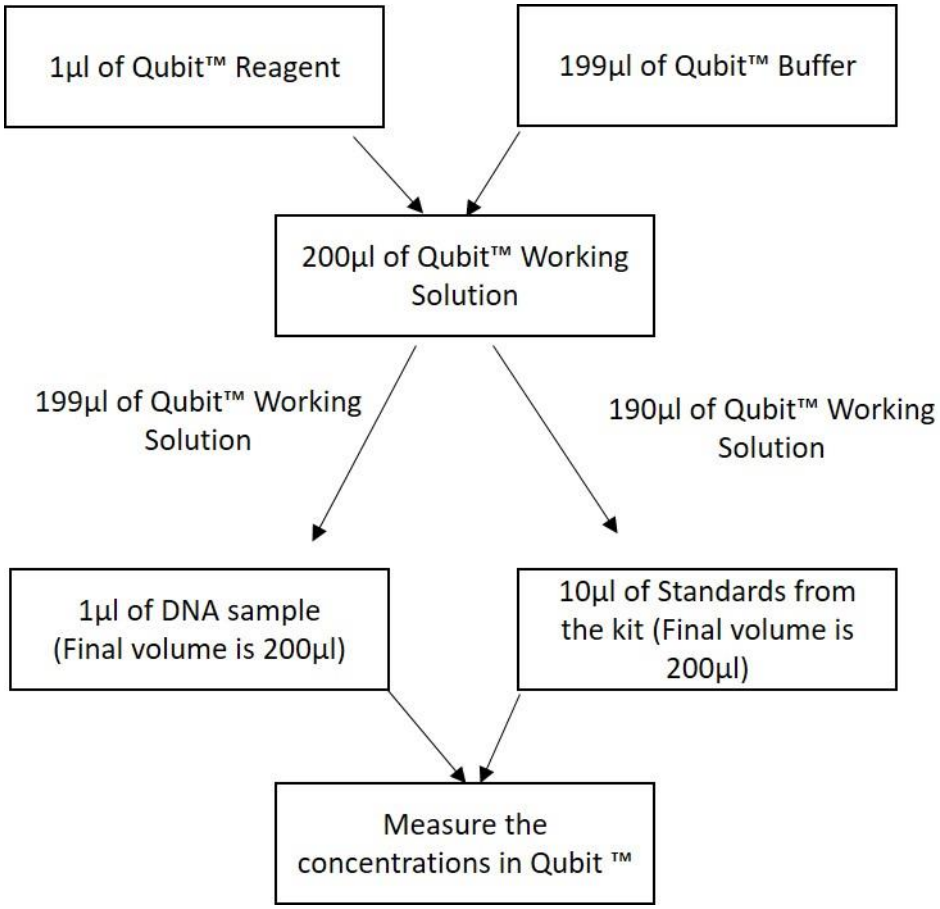

Figure S5- 1: Flow chart for measuring DNA concentrations of root nodules using Qubit™ assay.

All the assay tubes should be vortexed for 2-3 seconds followed by incubation under room temperature for another 2 minutes. Then the readings can be taken using Qubit™ 2.0 fluorometer.

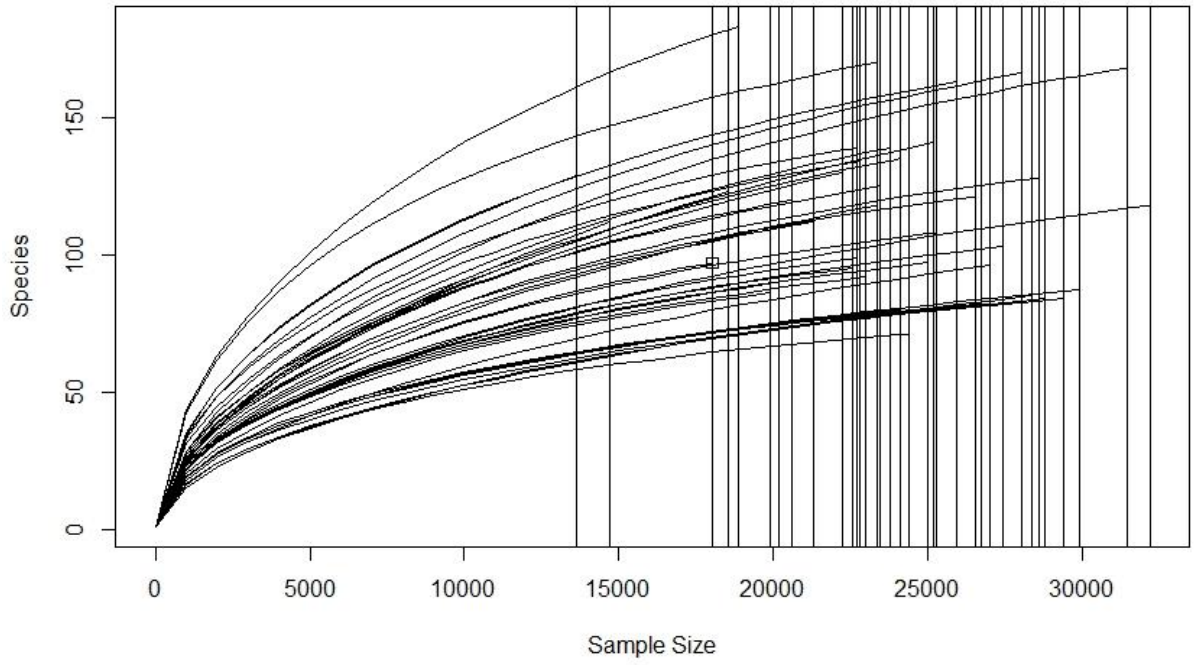

Figure S5- 2: Sample-effort curves of non-rhizobial endophyte (NRE) communities for each sample.

Table S5- 1: OTUs of NRE significantly associated with different rhizobial inoculation types (WSM1455, Competitors and Mix of 1455+ Competitors) under two watering treatments (well-watered -80%FWC and reduced watering- 60% FWC). The table includes type of rhizobial inoculation, watering treatment, the name of the OTU, indicator value (IV) range from 0 to 1 where highest values are associated with stronger indicators of the particular inoculation/watering condition, P value indicating the significance of occurrence of particular indicator OTU, the taxonomy of the OTU (based on the sequence comparison using BLAST), query cover with percentage of sequence cover, percentage ID (aligned residues). The Subject Sequence ID (SseqID) is the accession of the database sequence providing the best match.

Type of rhizobial inoculation	Watering Treatment	OTU ID	IV	*P*	Best matched taxa of NRE using BLAST			
					Taxanomy	Query cover	ID%	sseqID
WSM1455	well-watered	16Sall_OTUa_2296	0.61	0.04	*Fimbriimonas* sp.	100	98	1068363
WSM1455	reduced watering	16Sall_OTUd_278	0.63	0.03	*Thermobaculum terrenum*	99.6	85	4476741
		16Sall_OTUa_686	0.63	0.03	Solirubrobacterales	100	100	986601
Competitors	well-watered	16Sall_OTUb_5216	0.97	0.001	*Mucilaginibacter gracilis*	100	98	4397087
		16Sall_OTUb_43909	0.85	0.002	*Pedobacter*sp.	99.6	99	790094
		16Sall_OTUd_9926	0.67	0.01	*Pedobacter cryoconitis*	100	96	4397089
		16Sall_OTUa_59	0.66	0.02	*Planctomyces*	100	96	216002
		16Sall_OTUb_18243	0.66	0.02	Phycisphaerae	100	91	1002759
		16Sall_OTUb_5485	0.60	0.05	*Kaistobacter* sp.	100	97	4452571
		16Sall_OTUb_32312	0.59	0.05	*Dyella ginsengisoli*	98	97	693344
Competitors	reduced watering	16Sall_OTUa_6715	0.66	0.02	*Kineococcus xinjiangensis*	100	97	4431590
Mixed	well-watered	16Sall_OTUb_3030	0.94	0.001	*Acinetobacter johnsonii*	100	100	4481710
		16Sall_OTUe_18	0.85	0.001	*Pseudomonas viridiflava*	100	99	4364155
		16Sall_OTUa_3725	0.66	0.03	*Pseudonocardia*	100	99	157755
Mixed	reduced watering	16Sall_OTUa_1351	0.63	0.04	Nitrospirales 0319-6A21	100	100	4435512
		16Sall_OTUd_13055	0.63	0.03	*Salinispora*	100	98	4483546